Fields VIROLOGY

VOLUME 4: Fundamentals

SEVENTH EDITION

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David M. Knipe

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Wolters Kluwer

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Preface

In the early 1980s, Bernie Fields originated the idea of a virology reference textbook that combined the molecular aspects of viral replication with the medical features of viral infections. This broad view of virology reflected Bernie's own research, which applied molecular and genetic analyses to the study of viral pathogenesis, providing an important part of the foundation for the field of molecular pathogenesis. Bernie led the publication of the first three editions of Virology, but he unfortunately died soon after the third edition went into production. The third edition became *Fields Virology* in his memory, and it is fitting that the book continues to carry his name. A number of changes and enhancements have now been introduced with the seventh edition of *Fields Virology*. The publication format of *Fields Virology* has been changed from a once every 5-6 years, two volume book to an annual publication that comprises approximately one-fourth of the chapters organized by category. The annual publication provides both a physical book volume and importantly an eBook with an improved platform. Using an eBook format, our expectation is that individual chapters can be easily updated when major advances, outbreaks, etc., occur. The editorial board organized the four-volume series for the seventh edition to consist of volumes on Emerging Viruses, DNA Viruses, RNA Viruses, and Fundamental Virology, to be published on an annual basis, with the expectation that the topics will cycle approximately every 4 years creating an annualized, up-to-date publication. Each volume contains approximately 20 chapters. The first three volumes of this seventh edition of *Fields Virology*, entitled Emerging Viruses, DNA Viruses, and RNA Viruses were published in 2020, 2021, and 2022, respectively. This fourth volume, Fundamental Virology, has been principally edited by L. W. Enquist, Peter M. Howley, and David M. Knipe with valuable input from all of the Associate Editors. We wish to thank Patrick Waters of Harvard Medical School and all of the editorial staff members of Wolters Kluwer for all their important contributions to the preparation of this book.

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Introduction

Fundamental Virology is the fourth volume of the seventh edition of *Fields Virology*. The first three volumes, Emerging Viruses, DNA Viruses, and RNA Viruses were published in 2020, 2021, and 2022, respectively. There have been continued rapid advances in virology since the sixth edition that was published in 2013, and all the chapters in this Fundamental Virology volume are either completely new or have been significantly updated to reflect these advances. Major advances in basic virology since the last edition have been included in this volume.

This volume covers basic aspects of virology providing a historical context as well as fundamental principles, virus entry and uncoating, viral replication and assembly, and host immune responses to virology. There are chapters on viruses of insects, plants, bacteria, as well as chapters on prions as infectious agents. New chapters included in this seventh edition of *Fields Virology* are Metabolism and Viral Infection and Evolution of Viral Proteins. This Fundamental Virology volume is appropriate for use as a textbook for graduate students or advanced undergraduate students in virology courses, often when supplemented with individual virus chapters from Volumes 1-3 of this seventh edition.

We are grateful to L. W. Enquist, Jeff I. Cohen, Blossom Damania, Eric O. Freed, and Sean P. J. Whelan who joined us as the senior editors and participated in putting this volume together. We also are thankful to the chapter authors who have updated their chapters for this volume and to the new authors who have joined us in this continued endeavor to provide a comprehensive resource in basic virology.

Peter M. Howley
David M. Knipe

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CHAPTER 1

Virology: From Contagium Fluidum to Virome

L. W. Enquist • Vincent R. Racaniello

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The virome: how many viruses are there and where are they?

Pathogen discovery

Virology has had a remarkable history. Even though humans did not realize viruses existed until the late 1880s, viral infections have shaped the history and evolution of life on the planet. Viruses are likely to infect all living organisms, even though some have not yet been discovered. Not only do viral infections shape the evolution of their hosts but their hosts also influence the evolution of viruses. The consequences of viral infections not only altered human history but also have powerful effects on the entire ecosystem. As a result, virologists have gone to extraordinary lengths to study, understand, and in some cases attempt to eradicate these agents. It is noteworthy that just as the initial discovery of viruses required new technology (porcelain filters), uncovering the amazing biology underlying viral infections has gone hand in hand with new technology developments. Indeed, virologists have elucidated new principles of life processes and have been leaders in promoting new directions in science. For example, many of the concepts and tools of molecular and cell biology have been derived from the study of viruses and their host cells. This chapter is an attempt to review selected portions of this history as it relates to the development of new concepts in virology. A compendium of milestones in virology history is found in Table 1.1.

TABLE 1.1 Milestones in virology

Early observations and interventions

4000 BC: Visual evidence of rabies and polio in ancient art and artefacts
1796: Cowpox lesions used to vaccinate against smallpox (Jenner)
1885: Development of rabies vaccine (Pasteur, Roux)

The birth of virology

1882: Transmission of tobacco mosaic disease with cell-free extracts (Mayer)
1892: Description of filterable infectious agent (TMV) (Ivanovsky)
Discovery of virus neutralization (vaccinia) (Sternberg)
1898: Concept of the virus as a contagious element (TMV) (Beijerinck)
First animal virus (FMDV) (Loeffler, Frosch)
1901: First human virus (yellow fever virus) (Reed, Carroll, Agramonte, Lazear, Finlay)
1908: First leukemia-causing virus (Ellerman, Bang)
1909: Discovery of poliovirus (Landsteiner, Popper)
1911: First solid tumor virus (RSV) (Rous)
1913: Virus cultivation in tissue culture (VV) (Steinbault, Israeli, Lambert)
1915: Discovery of bacterial viruses (bacteriophages) (Twort, d'Hérelle)
1917: Development of the plaque assay and discovery of the particulate nature of viruses (bacteriophage) (d'Herelle)

1930–1950: Pathogen discovery and the birth of molecular biology

1931: Virus propagation in embryonated chicken eggs (Woodruff, Goodpasture)
Discovery of first influenza virus (swine influenza) (Shope)
1933: Discovery of human influenza virus (Smith)
1935: TMV crystallized (Stanley)
1936 TMV comprised of nucleoprotein and the nucleic acid is RNA (Bawden, Pirie)
1938: Yellow fever vaccine (Theiler)
Electron microscopy of viruses (TMV) (von Borries, Ruska, Ruska)
1939: One-step growth cycle (bacteriophage) (Ellis, Delbrück)
1941: First virus-associated enzymes (influenza virus) (Hirst)
1943: Genetic origins of mutations (bacteriophage) (Luria, Delbruck)
1948: Poliovirus replication in nonneuronal cell cultures (Enders, Weller, Robbins)
1949 Discovery of lysogeny and induction (bacteriophage) (Lwoff, Siminovitch, Kjeldgaard)

The remarkable 1950s

1951: Bacteriophage λ discovered (Lederberg)
1952: Animal virus plaque assay (poliovirus) (Dulbecco)
Discovery that viral genome is nucleic acid (Hershey, Chase)
Transduction of genetic information by bacteriophage (Zinder, Lederberg)
1953: Discovery of host-controlled restriction and modification (Luria, Bertani, Weigle)
1954: Development of polio vaccines (Salk, Sabin)
1955: Human cell culture (HeLa cells) (Gey)
In vitro assembly of infectious virus (TMV) (Fraenkel-Conrat, Williams)
1956: Discovery of mRNA in bacteriophage infection (Volkin, Astrachan, Brenner, Jacob, Meselson)
Virus particles composed of identical subunits (Watson, Crick)
RNA can carry genetic information (TMV) (Schramm, Fraenkel-Conrat, Williams)
1957: Discovery of interferon (Isaacs, Lindemann)
1958: Bacteriophage λ regulation paradigm (Pardee, Jacob, Monod, Lwoff)

The spectacular 1960s

1960: Discovery of SV40 (Sweet and Hilleman)
Triplet nature of the genetic code demonstrated (bacteriophage) (Crick)
Nonsense codons elucidated (bacteriophage) (Campbell, Epstein, Bernstein)
1962: Studies of virus structure (Klug, Caspar)
1964: Demonstration of the colinearity of gene with polypeptide chain (bacteriophage) (Brenner)
First human tumor virus (EBV) (Epstein, Barr, Burkitt)
1965: Autocatalytic *in vitro* synthesis of bacteriophage DNA (Spiegelman)
1967: Discovery of hepatitis B virus (Blumberg)
Isolation of bacteriophage λ repressor (Ptashne)
Discovery of viroids (Diener)
First virion-associated polymerase (VV) (McAuslan, Kates)
Discovery of the first filovirus (Marburg) (Siegert, Slenczka, Martini, Kissling, Robinson, Murphy, and others)

Ten highly productive years; the advance of recombinant DNA technology

1970: Discovery of retroviral reverse transcriptase (Temin, Baltimore)
1971: RNA polyadenylation (Darnell, Edmonds)
1972: First recombinant DNA molecules (phage λ, SV40) (Berg)
1973: First restriction map (SV40) (Nathans)
Major histocompatibility locus restriction of viral antigens (Doherty, Zinkernagel)
1974: First transgenic mouse (SV40) (Mintz)
Phage lambda vectors for recombinant DNA technology (Blattner, Davis, Enquist, Murray)

1975:	Discovery of mRNA capping (Shatkin, Moss)
1976:	First RNA virus genome sequenced (bacteriophage MS2) (Fiers)
	Demonstration that retroviral oncogenes are derived from cells (J. M. Bishop, Varmus)
1977:	First DNA virus genomes sequenced (ϕX174, SV40) (Sanger, Fiers, Weissman)
	RNA splicing discovered (adenovirus) (Roberts, Sharp)
	Discovery of tumor suppressor p53 (SV40) (Levine, Crawford)
	First virus crystal structure (TBSV) (Harrison)
1979:	World Health Organization declares smallpox eradicated
	First *in vitro* replication of viral DNA (adenovirus, SV40) (Kelly, Hurwitz, Stillman)
	First *in vitro* mRNA transcription system (adenovirus) (Roeder)
	First highly active template-specific RNA-dependent RNA polymerase from a eukaryotic source (BMV) (Hall)
	Discovery of tyrosine kinases (Hunter, Erikson, Eckhart)
The 1980s; major technology advances and four Nobel Prizes in 2 years	
1980:	First human retrovirus (HTLV-1) (Gallo)
1981:	First infectious molecular clones of an RNA virus (poliovirus) (Baltimore, Racaniello)
	Transcriptional enhancers discovered (Chambon, Khoury)
	Identification of mammalian transcription factors (MMTV, SV40) (Yamamoto, Tjian)
	Insertional activation of cellular oncogenes by retroviruses (Hayward, Astrin)
	Identification of polyadenylation signal (Shenk)
	Discovery of the Cre/lox recombination system in phage P1 (Sternberg)
	Structure of first viral envelope protein (Wiley, Skehel, Wilson)
1982:	Development of antiviral and other drugs (Elion, Hitchings)
	Definition of prions (Prusiner)
1982	Recombinant virus as vaccine for unrelated pathogens (Moss, Paoletti)
1983:	High-risk human papillomaviruses identified and linked to cervical cancer (zur Hausen)
	Discovery of AIDS virus (HIV) (Montagnier, Barre-Sinoussi, Gallo)
1984:	Discovery of nuclear localization signals (Smith, Butel)
	First infectious, multicomponent virus produced from cloned DNA (BMV) (Ahlquist)
1986:	First recombinant viral vaccine (HBV)
	Generation of transgenic virus-resistant plants (TMV) (Beachy)
	Discovery of hammerhead ribozymes (TRSV, ASV) (Bruening, Symons)
1988:	Discovery that DNA virus oncogenes products bind cellular tumor suppressor proteins (adenovirus, SV40, HPV) (Harlow, Weinberg, Livingston, Howley)
	First ribozyme with engineered specificity (Haseloff, Gerlach)
	Discovery of internal ribosome entry sites (poliovirus) (Wimmer, Sonenberg)
1989:	Discovery of hepatitis C virus (Houghton)
1990:	Transgenic mouse model for viral infection (poliovirus) (Ren and colleagues)
Technology and pathogen discovery as we enter the 21st century	
1990:	First human gene therapy with a retrovirus vector (Anderson, Blaese)
1991:	Discovery of viral antiapoptotic proteins (baculovirus) (Miller)
1994	Kaposi sarcoma herpesvirus discovered (HHV-8) (Moore, Chang)
1995:	Development of HAART treatment for AIDS
1998:	Gene silencing by double-stranded RNA, an antiviral response (Fire, Mello)
	Plant virion used for the synthesis of nanoparticles (Young)
	Discovery that plant viruses encode suppressors of RNAi (Vance, Baulcombe)
2002:	Severe acute respiratory syndrome (SARS) worldwide outbreak and containment
2003	Discovery of mimivirus (Raoult, Claverie)
2005:	Reconstruction and sequencing of the 1918 influenza virus genome (Palese, Garcia-Sastre, Tumpey, Taubenberger)
	Hepatitis C virus propagated in tissue culture (Chisari, Rice, Wakita)
2006:	Vaccine against human papillomavirus, the first vaccine designed to prevent human cancer
2008–2015:	Planned global eradication of poliomyelitis (WHO)
2010	Vertebrate genomes carry ancient viral genomes (Skalka, Bely; Taylor, Leach, Bruenn)
2011	Rinderpest virus declared eradicated
2012	Development of CRISPR technology for gene editing (Doudna, Chapentier, Zheng)
2013	Approval of telaprevir and boceprevir, first direct-acting antiviral drugs for hepatitis C virus
2017	Development of cryoelectron microscopy (Dubochet, Frank, Henderson)
2017	Delivery of CAR-T cells by retroviral vector approved by FDA
2017	Luxturna, an adenovirus-associated virus vector, approved by FDA for treatment of blindness
2020	WHO declares the COVID-19 pandemic
2020	First COVID-19 vaccines deployed after development in <1 year

Discoveries recognized by a Nobel Prize are highlighted in red.

See also[54] and A. Murphy's, online review: The Foundations of Medical and Veterinary Virology: Discoverers and Discoveries, Inventors and inventions, developers and technologies. http://talk.ictvonline.org/files/ictv_documents//documents/633.aspx

Abbreviations: ASV, avocado sunblotch viroid; BMV, brome mosaic virus; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; HPV, human papillomavirus; TBSV, tomato bushy stunt virus; TMV, tobacco mosaic virus; TRSV tobacco ringspot virus; SV40, simian virus; FMDV, foot and mouth disease virus; WHO, World Health Organization; MHC, major histocompatibility complex; RSV, Rous sarcoma virus.

The Concept of Viruses as Particulate Infectious Agents

A diverse microbial world of bacteria, fungi, and protozoa had been widely accepted by the last half of the 19th century. An early proponent of the germ theory of disease was the noted German anatomist Jacob Henle of Gottingen (the discoverer of Henle loop in kidneys and the grandfather of 20th-century virologist Werner Henle). He hypothesized in 1840 that specific diseases were caused by infectious agents that were

too small to be observed with the light microscope. But he had no evidence for such entities, and consequently his ideas were not generally accepted. It would take the work of Louis Pasteur and Henle's student, Robert Koch, before it became evident that microbes could cause diseases.

As early as 1728, the term virus was used to describe an agent that causes infectious disease. Long before bacteria, fungi, and viruses were identified, it was known that some diseases could be transmitted from person to person. The nature of such agents was unknown, but because the word virus comes from the Latin meaning a poison, we can surmise that the agents were thought to be liquids.

In the 1800s, the work of Pasteur, Lister, and Koch founded the field of microbiology, leading to the germ theory of disease: microbes could cause infectious diseases. Louis Pasteur (1822–1895) studied fermentation by different microbial agents. From his work, he concluded that "different kinds of microbes are associated with different kinds of fermentations" and he soon extended this concept to diseases. In fact, Pasteur thought that all infectious diseases were caused by microbes, as suggested by his statement that "every virus is a microbe." Pasteur's reasoning strongly influenced Robert Koch (1843–1910), a student of Jacob Henle and a country doctor in a small German village. He developed solid media to isolate colonies of bacteria to produce pure cultures and stains to visualize the microorganisms. With these tools in hand, he identified the bacterium that causes anthrax (*Bacillus anthracis*, 1876) and tuberculosis (*Mycobacterium tuberculosis*, 1882). Joseph Lister (1827–1912), a professor of surgery in Glasgow, had heard about Pasteur's work and surmised that a sterile field should be maintained during surgery. Although many other scientists of that day contributed tools and concepts, it was principally Pasteur, Lister, and Koch who put together a new experimental approach for medical science.

These observations led Robert Koch to formalize some of Jacob Henle's original ideas for defining whether a microorganism is the causative agent of a disease. Koch's postulates state that (1) the organism must be regularly found in the lesions of the disease, (2) the organism must be isolated in pure culture, (3) inoculation of such a pure culture of organisms into a host should initiate the disease, and (4) the organism must be recovered once again from the lesions of the host. By the end of the 19th century, these concepts outlined an experimental method that became the dominant paradigm of medical microbiology. It was only when these rules broke down and failed to yield a causative agent that the concept of a virus as an infectious particle was born.

A key point in the history of virology was the development in 1884 by Charles Chamberland, an associate of Pasteur, of a porcelain filter that could retain bacteria. The filters were initially used to provide bacteria-free water for the laboratory. Pasteur, working on the agent of rabies, found that it could pass through such filters and thought it to be unusually small.

Later Ivanovsky and Beijerinck showed that the agent of tobacco mosaic disease to be small enough to pass through filters. Beijerinck had the important insight that the pathogen could only be propagated in plants not in broth as was known for bacteria. He called it a "contagious living fluid." After this work, many similar agents were found and the name "filterable virus" was applied to distinguish them from infectious agents that were retained on the filter.

At this point, the distinctions between bacteria and viruses were on the basis of filterability and reproduction in broth. The exact nature of "filterable viruses" was obscure. Four subsequent observations were essential in leading to our current concept of viruses.

Bacteriophages were discovered in 1917 and found to cause clear spots, or plaques, on lawns of bacteria growing on agar plates. Such behavior did not fit in with the previously "fluid" nature of viruses. Bacteriophages were visualized by the electron microscope for the first time in 1939, proving beyond all doubt that they were particulate.

Crystals of the tobacco mosaic disease agent were produced by Stanley in 1935, which to chemists proved that filterable viruses were not only chemicals but could be made pure and homogeneous. To Stanley, this observation meant that filterable viruses were "infectious proteins," a conclusion he reached by ignoring the small amounts of phosphate present in his crystals. He was wrong about tobacco mosaic virus (TMV), whose RNA was later shown to be infectious, but many years later infectious proteins (prions) were discovered.

The last important observation on the road to virus was the development of the one-step growth curve of bacteriophages by Ellis and Delbruck in 1939. They noted that when viruses were added to bacteria, there was initially a lag phase during which no new infectious viruses were produced. This lag phase contrasted with observations made with bacteria: when added to broth, they immediately began to divide. This result showed that viruses were not simply miniature bacteria. We now know that the lag phase is a period during which new viral components are being synthesized, which are later assembled to form infectious particles. Bacteria reproduce by binary fission, and viruses reproduce by making new parts and assembling them.

As a consequence of these observations, the word "filterable" was dropped and these agents became simply "viruses" as their distinction from bacteria and other infectious agents became clear. And that is how we came to today's definition of virus, which began as a catchall for all agents of infectious diseases.

The Birth of Virology

Pathogen Discovery, 1886–1903

Adolf Mayer (1843–1942) was a German agricultural chemist and director of the Agricultural Experiment Station at Wageningen in The Netherlands when he was asked to investigate a disease of tobacco. He named the affliction tobacco mosaic disease after the dark and light spots that appeared on infected leaves (Fig. 1.1). To investigate the nature of the disease, Mayer inoculated healthy plants with the juice extracted from diseased plants by grinding up the infected leaves in water. Mayer reported that, "in nine cases out of ten (of inoculated plants), one will be successful in making the healthy plant . . . heavily diseased".[122] Although these studies established the infectious nature of the tobacco mosaic disease, neither a bacterial nor a fungal agent could consistently be cultured or detected in these extracts, so Koch's postulates could not be satisfied. In a preliminary communication in 1882,[121] Mayer speculated that the cause could be a "soluble, possibly enzymelike contagium, although almost any analogy for such a supposition is failing in science." Later Mayer concluded that the mosaic disease "is bacterial, but that the infectious forms have not yet been isolated, nor are their forms and mode of life known".[122]

FIGURE 1.1 **Lesions on a tobacco leaf infected with tobacco mosaic virus**. Adolph Mayer was the first to describe the pattern of *light* and *dark green areas* on infected leaves in 1886. Mayer showed that lesions could be transmitted from one plant to another using extracts produced from an infected leaf.

A few years later, Dimitri Ivanofsky (1864–1920), a Russian scientist working in St. Petersburg, was commissioned by the Russian Department of Agriculture to investigate the cause of a tobacco disease on plantations in Bassarabia, Ukraine, and the Crimea. Ivanofsky repeated Mayer's observations by showing that the sap of infected plants contained an agent that could transmit the disease to healthy plants. But he added an important step—before the inoculation step, he passed the infected sap through a Chamberland filter (Fig. 1.2). This device, made of unglazed porcelain and perfected by Charles Chamberland, one of Pasteur's collaborators, contained pores small enough to retard most bacteria. Ivanofsky reported to the Academy of Sciences of St. Petersburg on February 12, 1892 that "the sap of leaves infected with tobacco mosaic disease retains its infectious properties even after filtration through Chamberland filter candles".[90]

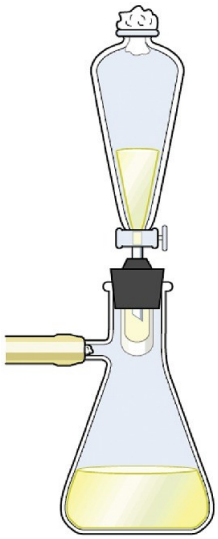

FIGURE 1.2 **Chamberland filter**. These filters, first used to demonstrate the filterable nature of viruses, are made of diatomaceous earth pressed into the form of a hollow candle. The fluid to be filtered is placed into the opening at the top using a funnel, and the filtrate, containing viruses, is collected in the flask.

Ivanofsky, like Mayer before him, failed to culture an organism from the filtered sap and could not satisfy Koch's postulates. Consequently, he suggested that a toxin (not a living, reproducing substance) might pass through the filter and cause the disease. As late as 1903, when Ivanofsky published his thesis,[91] he still believed that he had been unable to culture the bacteria that caused this disease. Bound by the dogma of Koch's postulates, Ivanofsky could not make a conceptual leap. It is, therefore, not surprising that Pasteur, who worked on the rabies vaccine[135] at the same time (1885), never investigated the unique nature of the infectious agent.

The conceptual leap was provided by Martinus Beijerinck (1851–1931), a Dutch soil microbiologist who collaborated with Adolf Mayer at Wageningen. Unaware of Ivanofsky's work, in 1898, he independently found that the sap of infected tobacco plants could retain its infectivity after passage through a Chamberland filter. But he also showed that the filtered sap could be diluted and regain its "strength" after replication in living, growing tissue of the plant. This observation showed that the agent could reproduce (therefore, it was not a toxin) but only in living tissue, not in the cell-free sap of the plant. Suddenly it became clear why others could not culture the pathogen outside its host. Beijerinck called this agent a contagium vivum fluidum,[10] or a contagious living liquid. He sparked a 25-year debate, summarized above, about whether these novel agents were liquids or particles. This conflict was resolved when d'Herelle developed the plaque assay in 1917[33] and when the first electron micrographs were taken of TMV in 1939.[99]

Mayer, Ivanofsky, and Beijerinck each contributed to the development of a new concept: a novel organism smaller than bacteria—an agent defined by the pore size of the Chamberland filter—which could not be seen in the light microscope and could multiply only in living cells or tissue. The term virus (from the Latin for slimy liquid or poison[85]) was at that time used interchangeably for any infectious agent, and so the agent of tobacco mosaic disease was called tobacco mosaic virus, TMV. The literature of the first decades of the 20th century often referred to these infectious entities as filterable agents, and this was indeed the operational definition of viruses. Sometime later, the term virus became restricted in use to those agents that fulfilled the criteria developed by Mayer, Ivanofsky, and Beijerinck.

Shortly after this pioneering work on TMV, the first filterable agent from animals was identified by Loeffler and Frosch—foot and mouth disease virus.[113] The first human virus discovered was yellow fever virus (1901), by Walter Reed and his team in Cuba.[144]

Plant Viruses and the Chemical Period: 1929–1956

For the next 50 years, TMV played a central role in research that explored the nature and properties of viruses. With the development of techniques to purify proteins in the first decades of the 20th century came the appreciation that the infectious material that passed through filters was proteins and so could be purified in the same way. Working at the Boyce Thompson Institute in Philadelphia, Vinson and Petre (1927–1931) precipitated infectious TMV (using an infectivity assay developed by Holmes[84]) from the crude sap of infected plants using selected salts, acetone, or ethyl alcohol.[178] They showed that the infectious material could move in an electric field, just as proteins did. At the same time, H. A. Purdy-Beale, also at the Boyce Thompson Institute, produced antibodies in rabbits that were directed against TMV and could neutralize the infectivity of this agent.[140] This observation was taken as further proof of the protein nature of viruses, although it was later realized that antibodies recognize chemicals other than proteins. With the advent of purification procedures for viruses, both physical and chemical measurements became possible. The strong flow birefringence of purified preparations of TMV was interpreted (correctly) to show an asymmetric particle or rod-shaped particle.[165] Max Schlesinger,[152] working on purified preparations of bacteriophages in Frankfurt, Germany, showed that the infectious particles were composed of proteins and also contained phosphorus and ribonucleic acid. This observation led to the first suggestion that virus particles were composed of nucleoproteins. The crystallization of TMV in 1935 by Wendell Stanley,[158] working at the Rockefeller Institute branch in Princeton, New Jersey, brought this infectious agent into the world of the chemists. Within a year, Bawden and Pirie[8,9] had demonstrated that crystals of TMV contained 0.5% phosphorus and 5% RNA. The first "view" of a virus came from x-ray crystallography using these crystals to show rods of a constant diameter aligned in hexagonal arrays containing RNA and protein.[16] The first electron micrographs of any virus were of TMV, and they confirmed that the virus particle is shaped like a rod[99] (Fig. 1.3).

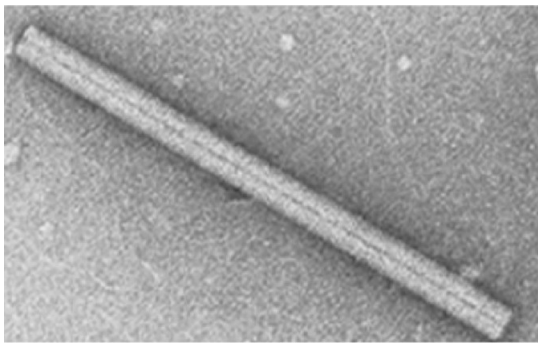

FIGURE 1.3 **Electron micrograph of the tobacco mosaic virus particle**. The virion is composed of a naked, helical nucleocapsid composed of multiple copies of a single viral protein complexed with the viral RNA genome.

The x-ray diffraction patterns[16] suggested that TMV was built from repeating subunits. These data and other considerations led Crick and Watson[32] to realize that most simple virus particles had to consist of one or a few species of identical protein subunits. By 1954–1955, techniques had been developed to dissociate TMV protein subunits, allowing reconstitution of infectious TMV from its RNA and protein subunits[63] and leading to an understanding of the principles of virus self-assembly.[25]

The concept that virus particles contained genetic information emerged as early as 1926, when H. H. McKinney reported the isolation of "variants" of TMV with a different plaque morphology that bred true and could be isolated from several geographic locations.[123,124] Seven years later, Jensen confirmed McKinney's observations[96] and showed that the plaque morphology phenotype could revert. Avery's DNA transformation experiments with pneumococcus[96] and the Hershey-Chase experiment with bacteriophages[80] both demonstrated that DNA was genetic material. TMV had been shown to contain RNA, not DNA, and in 1956, this nucleic acid was shown to be infectious. RNA, therefore, comprised the genetic material of the virus and was the first demonstration that RNA could be a genetic material.[63,70] Studies on the nucleotide sequence of TMV RNA confirmed codon assignments for the genetic code, adding clear evidence for the universality of the genetic code. These studies helped to elucidate the mechanisms of mutation by diverse agents.[62] Research on TMV and related plant viruses has contributed significantly to both the origins of virology and its development as a science.

Bacteriophages

Early Years: 1915–1940

Frederick W. Twort was superintendent of the Brown Institution in London when he discovered viruses of bacteria in 1915. In his research, Twort was searching for variants of vaccinia virus (the smallpox vaccine virus) that would replicate in simple defined media outside living cells. In one of his experiments, he inoculated nutrient agar with an aliquot of the smallpox vaccine (which essentially was lymph fluid from vaccinia pustules). The virus failed to replicate, but bacterial contaminants flourished on the agar medium. Twort noticed that some of these bacterial colonies changed visibly with time and became "watery looking" (ie, more transparent). The bacteria within these colonies were apparently dead, as they could no longer form new colonies on fresh agar plates. He called this phenomenon "glassy transformation." Simply adding the glassy transforming principle could rapidly kill a colony of bacteria. It readily passed through a porcelain filter, could be diluted a million-fold, and when placed upon fresh bacteria would regain its strength, or titer.[173–175]

Twort published these observations in a short note[173] in which he suggested that a virus of bacteria could explain glassy transformation. He then went off to serve in World War I, and when he returned to London, he did not continue this research.

While Twort was puzzled by glassy transformation, Felix d'Herelle, a Canadian medical bacteriologist, was working at the Pasteur Institute in Paris. When a *Shigella* dysentery infection devastated a cavalry squadron of French soldiers just outside of Paris in August 1915, D'Herelle readily isolated and cultured the dysentery bacillus from filtered fecal emulsions. The bacteria multiplied and covered the surface of his agar plates, but occasionally d'Herelle observed clear circular spots devoid of growth. He called these areas taches vierges, or plaques. He followed the course of an infection in a single patient, noting when the bacteria were most plentiful and when the plaques appeared.[33,34] Plaques appeared on the fourth day after infection and killed the bacteria in the culture dish, after which the patient's condition began to improve.

D'Herelle found that a filterable agent, which he called a bacteriophage, was killing the *Shigella* bacillus. In the ensuing years, he developed fundamental techniques in virology that are utilized to this day, such as the use of limiting dilutions to determine the virus titer by plaque assay. He reasoned that the appearance of plaques showed that the virus was particulate, or "corpuscular," and not a liquid as Beijerinck had insisted. D'Herelle also found that if infectious material was mixed with a host cell and then subjected to centrifugation, the infectious material was no longer present in the supernatant fluid. He interpreted this to mean that the first step of a virus infection is attachment, or adsorption, of virus particles to the host cell. Furthermore, particle attachment occurred only when bacteria sensitive to infection were used, demonstrating that host specificity can be conferred at a very early step in infection. Lysis of cells and the release of infectious particles were also described in startlingly modern terms. D'Herelle clearly established many of the principles of modern virology.[34,35]

Although D'Herelle's bacteriophages lysed their host cells, by 1921 it had become apparent that under certain situations the bacteriophage and cell existed peacefully—a condition called lysogeny. In some experiments, it became impossible to separate the virus from its host. This condundrum led Jules Bordet of the Pasteur Institute in Brussels to suggest that the transmissible agent described by d'Herelle was nothing more than a bacterial enzyme that stimulates its own production.[22] Although incorrect, the hypothesis has remarkable similarities to modern ideas about prion structure and replication (see Chapter 22).

During the 1920s and 1930s, d'Herelle sought ways to use bacteriophages for medical applications, but he never succeeded. Interestingly, phage therapy (the use of bacteriophages to treat patients with bacterial infections) has seen renewed interest. To complicate matters, the basic research of the era was dominated by the interpretations of scientists with the strongest personalities. Although it was clear that there were many diverse bacteriophages, and that some were lytic while some were lysogenic, their interrelationships remained ill defined and contentious. The highlight of this period was the demonstration by Max Schlesinger that purified phages had a maximum linear dimension of 0.1 micron and a mass of about 4×10^{-16} g, and that they were composed of protein and DNA in roughly equal proportions.[151,152] In 1936, no one quite knew what to make of that observation, but over the next 20 years, it would begin to make a great deal of sense.

Phages and the Birth of Molecular Biology: 1938–1970

Max Delbrück was trained as a physicist at the University of Göttingen, and his first position was at the Kaiser Wilhelm Institute for Chemistry in Berlin. There he joined a diverse group of individuals actively discussing how quantum physics related to an understanding of heredity. Delbrück's interest in this area led him to develop a quantum mechanical model of the gene, and in 1937, he moved to the Biology Division at the California Institute of Technology to study genetics of Drosophila. Once there, he became interested in bacteria and their viruses and teamed up with another research fellow, Emory Ellis,[50] who was working with the T-even group of bacteriophages, T2, T4, and T6. Delbrück soon appreciated that these viruses were ideal for the study of how genes replicated and how genetic information could determine the structure and function of an organism. They replicated rapidly and offered simple quantitative assays. Delbruck brought the rigor of quantitative, well-defined experiments to virology. Bacteriophages were also viewed as model systems for understanding cancer viruses or even for understanding how a sperm fertilizes an egg and a new organism develops. Together with Ellis, Delbrück showed that when infections were synchronized, new virus particles were produced in one step (a burst),[51] in contrast to the multiplication of other organisms by binary fission. The elegant one-step growth curve experiment showed that a single infected bacterium liberates hundreds of phages synchronously after a half hour period during which viral infectivity was lost (Fig. 1.4). The one-step growth curve became the experimental paradigm of the phage group.

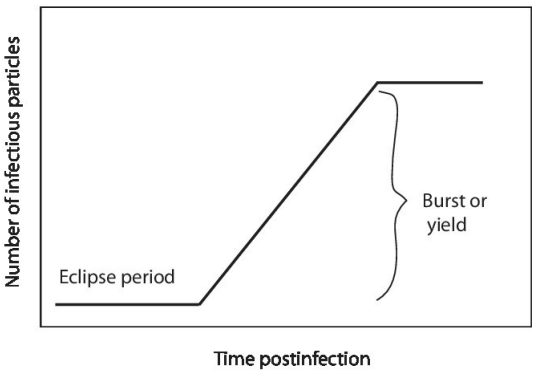

FIGURE 1.4 **One-step growth curve of bacteriophage**. The conclusion of Ellis and Delbrück that viruses reproduced in one step was drawn from the one-step growth curve experiment, in which an infected bacterium liberates phages synchronously after a half hour period (eclipse) during which viral infectivity is lost.

When World War II erupted, Delbrück remained in the United States (at Vanderbilt University) and met an Italian refugee, Salvador E. Luria, who had fled to America and was working at Columbia University in New York (on bacteriophages T1 and T2). After their encounter at a meeting in Philadelphia on December 28, 1940, they went to Luria's laboratory at Columbia where they spent 48 hours doing experiments with bacteriophages. These two scientists eventually established the "phage group," a community of researchers focused on using bacterial viruses as a model for understanding life processes. Luria and Delbrück were invited to spend the summer of 1941 at Cold Spring Harbor Laboratory, where they pursued research on phages. It is remarkable that a German physicist and an Italian geneticist joined forces during the war years to travel throughout the United States and recruit a new generation of biologists (Fig. 1.5).

FIGURE 1.5 **Max Delbrück and Salvador Luria at Cold Spring Harbor Laboratory**. Photograph taken during the 1953 Cold Spring Harbor Symposium on Quantitative Biology organized by Milislav Demerec. (From http://profiles.nlm.nih.gov/ps/retrieve/ResourceMetadata/QLBBJF. Courtesy of the Cold Spring Harbor Laboratory Archives, NY.)

When Tom Anderson, an electron microscopist at the RCA Laboratories in Princeton, New Jersey, met Delbrück, the result was the first clear pictures of bacteriophages.[117] At the same time, the first phage mutants were isolated and characterized.[115] By 1946, the first phage course was being taught at Cold Spring Harbor, and in March 1947, the first phage meeting attracted eight people. From these humble beginnings, the field of molecular biology emerged, which focused on the bacterial host and its viruses.

Developing the Modern Concept of Virology

The next 25 years (1950–1975) was an intensely productive period of bacteriophage research. Hundreds of virologists produced thousands of publications that covered three major areas: (1) lytic infection of *Escherichia coli* with the T-even phages; (2) the nature of lysogeny, using lambda phage; and (3) the replication and properties of several unique phages such as ϕX174 (single-stranded circular DNA), the RNA phages, and T7. This work set the foundations for modern molecular virology and biology.

The idea of examining, at the biochemical level, the events occurring in phage-infected cells during the latent period had come into its own by 1947–1948. Impetus for this work came from Seymour Cohen, who had trained first with Erwin Chargaff at Columbia University, studying lipids and nucleic acids, and then with Wendell Stanley working on TMV RNA. His research direction was established after taking Delbrück's 1946 phage course at Cold Spring Harbor, Cohen examined the effects of phage infection on DNA and RNA levels in infected cells using a colorimetric analysis.[30] The results showed a dramatic alteration of macromolecular synthesis in infected cells. This included cessation of RNA accumulation, which later formed the basis for detecting a rapidly turning over species of RNA and the first demonstration of messenger RNA (mRNA).[4] DNA synthesis also halted, but only for 7 minutes, followed by resumption at a 5- to 10-fold increased rate. At the same time, Monod and Wollman showed that the synthesis of a cellular enzyme, the inducible β-galactosidase, was inhibited after phage infection.[125] Based on these observations, the viral eclipse period (when infectivity was lost immediately after infection) was divided into an early phase, prior to DNA synthesis, and a late phase. More importantly, these results demonstrated that virus infection could redirect cellular macromolecular synthetic processes in infected cells.[31]

By the end of 1952, two experiments had a critical effect on virology. First, Hershey and Chase asked whether bacteriophage genetic information is DNA or protein. They differentially labeled viral proteins (with 35SO4) and nucleic acids (with 32PO4) and allowed the radioactive "tagged" particles to attach to bacteria. When they sheared the viral protein coats from the bacteria using a Waring blender, only DNA was associated with the infected cells.[80] This result proved that DNA had all the information needed to reproduce new virus particles. A year later, the structure of DNA was elucidated by Watson and Crick, a discovery that permitted full appreciation of the Hershey-Chase experiment.[180] The results of these two experiments formed a cornerstone of the molecular biology revolution.

While these blockbuster experiments were being carried out, G. R. Wyatt and S. S. Cohen were quietly making another seminal finding.[191] They identified a new base, hydroxymethylcytosine, in the DNA of T-even phages, which replaced cytosine. This began a 10-year study of how deoxyribonucleotides were synthesized in bacteria and phage-infected cells and led to the critical observation that bacteriophage infection introduces genetic information for a new enzyme.[59] By 1964, Mathews and colleagues had proved that hydroxymethylase does not exist in uninfected cells and must be encoded by the virus genetic material.[31] These experiments introduced the concept of early enzymes, utilized in deoxypyrimidine biosynthesis and DNA replication,[102] and provided biochemical proof that viral genomes encoded new information expressed as proteins in an infected cell. At the same time, phage genetics became extremely sophisticated, allowing mapping of the genes encoding these viral proteins. Perhaps the best example of genetic fine structure was done by Seymour Benzer, who carried out a genetic analysis of the rII A and B cistrons of T-even phages with a resolution of a single nucleotide (without doing any DNA sequencing!).[13] Studies on viral DNA synthesis, using phage mutants and cell extracts to complement and purify enzyme activities *in vitro*, contributed a great deal to our understanding of DNA replication.[1] A detailed genetic analysis of phage assembly, utilizing the complementation of phage assembly mutants *in vitro*, revealed

how complex structures are built using the principles of self-assembly.[45] The genetic and biochemical analysis of phage lysozyme helped to elucidate the molecular nature of mutations,[161] and the isolation of phage nonsense mutations (first called "amber" mutations) provided a clear way to study second-site suppressor mutations at the molecular level.[14] The circular genetic map of the T-even phages[161] was explained by the circularly permuted, terminally redundant conformation of these DNAs (giving rise to phage heterozygotes).[171]

The remarkable reprogramming of viral and cellular protein synthesis in phage-infected cells was dramatically visualized by an early use of electrophoresis and sodium dodecyl sulfate (SDS)-polyacrylamide gels.[104] These studies showed that viral proteins are made in a specific sequence of events. The underlying mechanism of this temporal regulation led to the discovery of proteins (sigma factors) that modified RNA polymerase and conferred specificity to gene expression.[73] The study of gene regulation at almost every level (transcription, RNA stability, protein synthesis, protein processing) was revealed from a set of original contributions derived from an analysis of phage infections.

Although this remarkable progress had begun with the lytic phages, no one knew quite what to make of the lysogenic phages. This situation changed in 1949 when André Lwoff began his studies at the Pasteur Institute with Bacillus megaterium and its lysogenic phages. By using a micromanipulator, Lwoff could show that single lysogenic bacteria divided up to 19 times before liberating infectious particles. No virions were detected when lysogenic bacteria were broken open by the investigator. But from time to time, a lysogenic bacterium spontaneously lysed and produced many infectious particles.[119] Many potential inducing agents were investigated, but ultraviolet light was very efficient. Indeed, exposing lysogenic bacteria to ultraviolet light efficiently induced production of infectious particles. This experiment began[120] to outline this curious relationship between a virus and its host. By 1954, Jacob and Wollman[93,94] at the Pasteur Institute had made the important observation that a genetic cross between a male, lysogenic bacterial strain (Hfr, lambda) and a nonlysogenic female recipient resulted in the induction of infectious particles after conjugation, a process they called zygotic induction. In fact, the position of the lysogenic phage (also called prophage) in the chromosome of its host *E coli* could be mapped by interrupting mating between two strains.[94] This experiment was crucial for our understanding of lysogenic viruses because it showed that the provirus behaved like a bacterial gene on a chromosome in a bacterium. It also was one of the first experimental results suggesting that the prophage genetic material was kept quiescent by negative regulation, which was lost as the chromosome passed from the lysogenic donor bacteria to the nonlysogenic recipient host. This conclusion helped Jacob and Monod to realize as early as 1954 that the "induction of enzyme synthesis and of phage development are the expression of one and the same phenomenon".[119] These experiments laid the foundation for the operon model and[180] the nature of coordinate gene regulation.

Although the structure of DNA was elucidated in 1953 and zygotic induction was described in 1954, the physical interaction between the bacterial chromosome and the viral genetic material in lysogeny was not clear. It often was referred to as the "attachment site" and literally thought of in those terms. The close relationship between a virus and its host was appreciated only when Campbell proposed the model for physical integration of lambda DNA into the bacterial chromosome,[26] based on the fact that the sequence of phage markers was different in the integrated state than in the replicative or vegetative state. The idea was that a circular lambda DNA molecule integrated by recombination between a specific site on the phage DNA with a specific site on the bacterial DNA. This integrated DNA was the prophage, which was transcriptionally silent. This model led to the isolation of the negative regulator or repressor of lambda, the understanding of why lysogens could not be infected by related bacteriophages (so-called immunity), and one of the early examples coordinated gene regulation.[139] The genetic analysis of the lambda bacteriophage life cycle is one of the great intellectual adventures in microbial genetics.[79] It deserves to be reviewed in detail by all students of molecular virology and biology. Other lysogenic *E coli* phages offered different paradigms that did not involve integration of viral DNA into host DNA. Bacteriophage P1 has a circular genome that is maintained in the cytoplasm as an episome and maintains stable inheritance by repressors and cytoplasmic replication.

The lysogenic phages[193] of *Salmonella typhimurium* such as P22 provided the first example of generalized transduction, whereas lambda provided the first example of specialized transduction.[128] The finding that virus particles could not only carry cellular genes but transfer those genes from one cell to another provided not only a method for fine genetic mapping but also a new concept in virology. As the genetic elements of bacteria were studied in more detail, it became clear that there was a remarkable continuum from lysogenic phages to episomes, transposons and retrotransposons, insertion elements, retroviruses, hepadnaviruses, viroids, and prions. Genetic information moves between viruses and their hosts to the point where definitions and classifications begin to blur.

The genetic and biochemical concepts that emerged from the study of bacteriophages stimulated the next phase of virology. The lessons of the lytic and lysogenic phages were often relearned and modified as the animal viruses were studied.

Animal Viruses

Cell Culture Technology and Discovery: 1898–1965

Once the concept took hold that viruses were particulate filterable agents, many diseased animal tissues were subjected to filtration to determine if a virus were involved. Filterable agents were found that were invisible in a light microscope and replicated only in living animal tissue. There were some surprises, such as the transmission of yellow fever virus by a mosquito vector,[144] specific visible pathologic inclusion bodies (virions and subviral particles) in infected tissue,[91,133] and even viral agents that can "cause cancer."[49,146] Throughout this early period (1900–1930), a wide variety of particulate animal infectious agents were found (see Table 1.1) and characterized with regard to their size (using the different pore sizes of filters), resistance to chemical or physical agents (eg, alcohol, ether), and pathogenic effects. Just based on these properties, it became clear that viruses were a very diverse group. Some were even observable in the light microscope (vaccinia in dark-field optics). Some were inactivated by ether, whereas others were not. Infectious agents that replicated in every tissue type were identified. Some were specific for a particular tissue. They could cause chronic or acute disease; they were persistent agents or recurred in a periodic fashion. Some caused cellular destruction or induced cellular proliferation. Some changed the morphology of the cells they infected. For the early virologists, unable to see their agents in a light microscope and often confused by this great diversity, many studies certainly required an element of faith. In 1912, S. B. Wolbach, an American pathologist, remarked, "It is quite possible that when our knowledge of filterable viruses is more complete, our conception of living matter will change considerably, and that we shall cease to attempt to classify the filterable viruses as animal or plant".[188]

The first way out of this early confusion was led by the plant virologists and the development of techniques to purify viruses and characterize both the chemical and physical properties of these agents (see previous section, The Plant Viruses and the Chemical Period: 1929–1956). The second path came from the studies with bacteriophages, where single cells infected with viruses in culture were much more amenable to experimental manipulation than were virus infections of whole animals. Whereas the plant virologists of that day were tethered to their

greenhouses, and the animal virologists were bound to their animal facilities, the viruses of bacteria were studied in Petri dishes and test tubes. Nevertheless, progress was made in the study of animal viruses one step at a time; from studying animals in the wild, to laboratory animals, such as the mouse[64] or embryonated chicken eggs,[189] to the culture of tissue, and then to single cells. Between 1948 and 1955, a critical transition converting animal virology into a laboratory science came in four important steps: Sanford and colleagues at the National Institutes of Health (NIH) overcame the difficulty of culturing single cells[149]; George Gey at Johns Hopkins Medical School cultured and passaged human cells for the first time and developed a line of immortal cells (HeLa) from a cervical carcinoma[69]; and Harry Eagle at the NIH developed an optimal medium for the culture of single cells.[44] In a demonstration of the utility of all these methods, Enders and his colleagues showed that poliovirus could replicate in a nonneuronal human explant of embryonic tissues.[53]

These ideas, technical achievements, and experimental advances had immediate effects on the field of virology. One of these was the development of the polio vaccine, the first ever produced in cell culture. From 1798 to 1949, all the vaccines in use (smallpox, rabies, yellow fever, influenza) had been grown in animals or embryonated chicken eggs. Poliovirus was grown in monkey kidney cells propagated in flasks.[81,110] The modern era of molecular virology began with the exploitation of cell culture. The first plaque assay for an animal virus in culture was done with poliovirus,[41] and it led to an analysis of poliovirus every bit as detailed and important as the contemporary work with bacteriophages. The simplest way to document this statement is for the reader to compare the first edition of General Virology by S. E. Luria in 1953[116] to the second edition by Luria and J. E. Darnell in 1967[118] and to examine the experimental descriptions of poliovirus infection of cells. The modern era of virology had arrived, and it would continue to be full of surprises.

The Molecular and Cell Biology Era of Virology

The history of virology has so far been presented chronologically or according to separate virus groups (plant viruses, bacteriophages, and animal viruses), which reflects the historical separation of these fields. In this section, the format changes as the motivation for studying viruses began to change. Virologists began to use viruses to probe questions central to understanding all life processes. Because viruses replicate in and are dependent on their host cells, they must use the rules, signals, and regulatory pathways of the host. By using viruses to probe these processes, virologists began to make contributions to all facets of biology. This approach began with the phage group and was continued by the animal virologists. The recombinant DNA revolution also took place during this period (1970 to the present), and both bacteriophages and animal viruses played a critical and central role in this revolution. For these reasons, the organization of this section focuses on the advances in cellular and molecular biology made possible by experiments with viruses. Some of the landmarks in virology since 1970 are listed in Table 1.1.

The Role of Animal Viruses in Understanding Eukaryotic Gene Regulation

Viral genetic material can be DNA or RNA, single stranded or double stranded.[156] How these forms of nucleic acid are replicated, how gene products are produced, and how they are packaged into infectious particles were and still are fundamental questions. The closed circular and superhelical nature of polyoma virus DNA was elucidated by Dulbecco and Vogt[42] and Weil and Vinograd.[181] The reason why the viral genome adopted this structure was first shown to be related to the packaging of simian vacuolating virus 40 (SV40) DNA into virus particles. The viral DNA is wound around nucleosomes during replication and is packaged into particles.[68] When the histones are removed, a DNA superhelix results. The structure of polyoma viral DNA served as an excellent model for the *E coli* genome[190] and mammalian chromosomes.[105,106]

Many elements of the eukaryotic transcription machinery have been elucidated with viruses. The first transcriptional enhancer element (acts in an orientation- and distance-independent fashion) was described in the SV40 genome,[74] as was a distance- and orientation-dependent promoter element.[108] The transcription factors that bind to the promoter, SP-1,[43] or to the enhancer element, such as AP-1 and AP-2, and which are essential to promote transcription along with the basal factors, were first described with SV40. AP-1 is composed of fos and jun family member proteins, demonstrating the role of transcription factors as oncogenes.[21] Indeed, the great majority of experimental data obtained for basal and accessory transcription factors come from *in vitro* transcription systems using the adenovirus major late promoter or the SV40 early enhancer-promoter.[182] Our present-day understanding of RNA polymerase III promoter recognition comes, in part, from an analysis of the adenovirus VA gene transcribed by this polymerase.[61]

Almost everything we know about the steps of mRNA processing began with observations made with viruses. The list is long but here are a few highlights: RNA splicing of new transcripts was first described in adenovirus-infected cells[15,28]; polyadenylation of mRNA was first observed with poxviruses,[97] the first viruses shown to have a DNA-dependent RNA polymerase in the virion[98]; the signal for polyadenylation of mRNA was identified using SV40[58]; the methylated cap structure found at the 5'-end of most mRNAs was first discovered on reovirus mRNAs[65]; and a remarkable discrimination for transport of viral and cellular mRNAs out of the nucleus was shown by the adenovirus E1B-55Kd protein.[137]

Our early understanding of translational regulation came from studies of virus infected cells. Recruitment of ribosomes to mRNAs was shown to be directed by the 5'-cap structure first discovered on reovirus mRNAs. The nature of the protein complex that allows ribosomes to bind the 5'-cap was elucidated in poliovirus-infected cells, because viral infection leads to cleavage of one of the components, eIF4G. Internal initiation of translation was discovered in cells infected with picornaviruses (poliovirus and encephalomyocarditis virus).[95,136] Interferon, discovered as a set of proteins that inhibited influenza virus replication, was subsequently found to induce the synthesis of many antiviral gene products that act on translational regulatory processes.[88] Similarly, the viral defenses against interferon by the adenovirus VA RNA provided unique insight to the role of eIF-2 phosphorylation events.[101] Mechanisms for producing more than one protein from a eukaryotic mRNA (there is no "one mRNA one protein" rule as described for bacteria) were discovered in virus-infected cells, including polyprotein synthesis, ribosomal frameshifting, and leaky scanning. Posttranslational processing of proteins by proteases, carbohydrate addition to proteins in the Golgi apparatus, phosphorylation by a wide variety of important cellular protein kinases, or the addition of fatty acids to membrane-associated proteins have all been profitably studied using viruses. Indeed, a good deal of our present-day knowledge of how protein trafficking occurs and is regulated in cells comes from the use of virus-infected cell systems. The field of gene regulation has derived many of its central tenets from the study of viruses.

Animal Viruses and the Recombinant DNA Revolution

The discovery of the enzyme reverse transcriptase[5,170] not only elucidated the replication cycle of retroviruses but also provided an essential

tool to convert RNA molecules to DNA, which could then be cloned and manipulated. The first restriction enzyme map of a chromosome was done with SV40 DNA, using the restriction enzymes HindII plus HindIII DNA.[36,37] The first demonstration of restriction enzyme specificity was carried out with the same viral DNA cleaved with EcoRI.[127,129] Some of the earliest DNA cloning experiments involved insertion of SV40 DNA into bacteriophage lambda DNA, or human β-hemoglobin genes into SV40 DNA, yielding the first mammalian expression vectors.[92] A debate about whether these very experiments were potentially dangerous led to a temporary moratorium on all such recombinant experiments following the scientist-organized Asilomar Conference. From the earliest experiments in the field of recombinant DNA, several animal viruses had been developed into expression vectors to carry foreign genes, including SV40,[72] retroviruses,[183] adenoviruses,[67] and adeno-associated virus.[148] Modern day strategies of gene therapy rely on some of these recombinant viruses. The gene for beta hemoglobin was first cloned using lambda vectors. The elusive hepatitis virus C (previously known as non-A, non-B) viral genome was cloned from serum using recombinant DNA techniques, reverse transcriptase, and lambda phage vectors.[27]

The study of RNA viruses was accelerated by recombinant DNA technology, particularly in the construction of "infectious clones." The RNA virus genome was converted into DNA by reverse transcriptase and the resulting DNA was cloned into appropriate vectors. When introduced into cells, the DNA was copied into RNA that was infectious.[142,168]

Animal Viruses and Oncology

Much of our present understanding of the origins of human cancers is a consequence of work on two major groups of animal viruses, retroviruses, and DNA tumor viruses (see Chapter 11). The first oncogene discovered was from the genome of Rous sarcoma virus. Remarkably, the virus had picked up a host gene that was involved in growth control.[159] Since those seminal studies, virologists have identified a wide variety of oncogenes that have been captured by retroviruses (see Chapter 8). Additional cellular oncogenes were identified when they were activated by insertion of retroviral proviral DNA in or near the gene.[75] When activated, these oncogenes are dominant and activate cell growth. The second group of genes that contribute to the origins of human cancers is the tumor suppressor genes that stop cell growth.[109] Two such genes (*p53* and *pRB*) are intimately associated with the so-called DNA tumor viruses that must replicate their DNA in cells that are not replicating. Genetic alterations at the p53 locus are the single most common mutations known to occur in human cancers—they are found in 50%-80% of all cancers.[111] The p53 protein was first discovered in association with the SV40 large T-antigen, a viral gene essential for viral DNA replication.[107,112] SV40, the human adenoviruses, and the human papillomaviruses all encode proteins that interact with and inactivate the functions of the retinoblastoma susceptibility gene product (Rb) and p53.[39,43,107,112,150,184,185] Our understanding of the roles of cellular oncogenes and the tumor suppressor genes in human cancers would be far less significant without the insight provided by studies with these viruses.

Viruses associated with cancers have provided some of the most extraordinary episodes in modern animal virology.[126] The recognition of a new disease and the unique geographic distribution of Burkitt lymphomas in Africa[17] (see Chapter 11) set off a search for viral agents that cause cancers in humans. From D. Burkitt[24] to Epstein, Achong, and Barr[56] to W. Henle and G. Henle,[78] the story of the Epstein-Barr virus and its role in several cancers, as well as in infectious mononucleosis, is a science detective story without rival. Similarly, the identification of adult T-cell leukemia, a new pathologic disease, in Japan by K. Takatsuki[176] led to the isolation of human T-cell leukemia virus (HTLV-1) by I. Miyoshi and Y. Hinuma.[138] It was soon realized that this virus had been identified previously by Gallo and his colleagues. Even with the virus in hand, there is still no satisfactory explanation of how this virus contributes to adult T-cell leukemia.

An equally interesting detective story concerns hepatocellular carcinoma and the discovery of hepatitis B virus. By 1967, S. Krugman and his colleagues[103] had strong evidence indicating the existence of distinct hepatitis A and B viruses, and in the same year B. Blumberg[20] had identified an unusual protein called the Australia antigen. Through a tortuous path, it eventually became clear that the Australia antigen was the coat protein of hepatitis B virus. Its detection was a predictable indictor for the presence of hepatitis B virus. Although this discovery ultimately freed the blood supply of this dangerous virus, Hilleman at Merck, Sharp, and Dohme and the Chiron Corporation (which later isolated the hepatitis C virus) went on to produce the first human vaccine that prevents hepatitis B infections and very likely hepatocellular carcinomas associated with chronic virus infections (see Chapter 15). The idea that a viral vaccine could prevent cancer (first proven with the Marek disease virus and T-cell lymphomas in chickens)[18,47] came some 82-85 years after the first discoveries of tumor viruses by Ellerman, Bang, and Rous.

Vaccines and Antivirals

Among the most remarkable achievements of the 20th century is the eradication of smallpox, a disease with a >2000-year-old history.[76] In 1966, the World Health Organization began a program to immunize all individuals who had encountered an infected person. This strategy was adopted because it simply was not possible to immunize entire populations. In October 1977, Ali Maolin of Somalia was the last person in the world to have a naturally occurring case of smallpox (barring laboratory accidents). Because smallpox has no animal reservoir and requires person-to-person contact for its spread, most scientists agree that we are free of this disease as a natural infection.[76] Consequently, most populations have not maintained immunity to the virus and the world's populations are becoming susceptible to infection. Many governments now fear the use of smallpox virus as a weapon of bioterrorism, and the debate continues over whether to destroy the two known stocks of smallpox virus in the United States and Russia.[77] As a consequence, the development of new, more effective vaccines and safe anti–smallpox virus drugs has risen high on the list of priorities for some countries and have already been stockpiled in the United States. It is paradoxical that humankind's most triumphant medical accomplishment is now tarnished by the specter of biowarfare.

The Salk and Sabin poliovirus vaccines were the first products to benefit from the cell culture revolution. In the early 1950s in the United States, just before the introduction of the Salk vaccine, about 21,000 cases of poliomyelitis were reported annually. Today, thanks to aggressive immunization programs, polio has been eradicated from most of the world (see Chapter 11).[132] However, complete eradication has proven to be difficult in some countries due to economic and political issues. With the substantial financial support of the Gates Foundation, there is hope that global immunization campaigns can lead to eradication of poliomyelitis from the planet.

The first viral vaccines deployed included infectious but attenuated viruses, inactivated virus preparations, and subunit vaccines comprising viral proteins and no viral nucleic acid. Both the Salk inactivated virus vaccine and the recombinant hepatitis B virus subunit vaccine were products of the modern era of virology. Today many new vaccine technologies are either in use or are being tested for future deployment.[3,23,153]

These include recombinant subunit vaccines, viruslike particle vaccines, viral antigens delivered in viral vectors comprising vaccinia virus or adenovirus, DNA plasmids that express viral proteins from strong promoters, and mRNA vaccines. Therapeutic vaccines boost the immune system using specific cytokines or hormones in combination with new adjuvants to stimulate immunity at specific locations in the host or to tailor the production of immune effector cells and antibodies. The first vaccines for smallpox were reported in the Chinese literature of the 10th century,[57] but up until a few years ago, progress and new approaches had been slow.

Although vaccines have been extraordinarily successful in preventing specific diseases, up until the 1960s, few natural products or chemotherapeutic agents that cured or reduced viral infections were known. That situation changed dramatically with the development of Symmetrel (amantadine) by Dupont in the 1960s as a specific influenza A virus drug. Soon after, acyclovir, an inhibitor of herpesviruses, was developed by Burroughs-Wellcome. Acylovir achieves its remarkable specificity because to be active, it must be phosphorylated by the viral enzyme thymidine kinase before it can be incorporated into viral DNA by the viral DNA polymerase. This drug blocks HSV-1 and HSV-2 replication after reactivation from latency and stopped a growing epidemic in the 1970s and 1980s (Chapter 14). The development of other nucleoside analogs that inhibit viral replication has led to many compounds effective against DNA viruses. Until the HIV epidemic, few drugs effective against RNA viruses other than the influenza A virus were known. That issue has changed dramatically in the past 10 years as many new antivirals effective against different RNA viruses, particularly HIV and HCV, have been discovered.

As natural products, the interferons are used successfully in the clinic for hepatitis B and C infections, cancer therapy, and multiple sclerosis. The interferons, novel cytokines, produced by infected cells were found while studying virus interference.[23,88,89] They modulate the immune response and continue to play an increasing role in the treatment of many clinical syndromes.

Virology and the Birth of Immunology

Edward Jenner was a British surgeon who is credited with making the first smallpox vaccine in 1796 and has also been called the "father of immunology." Jenner began a long tradition of virology providing seminal discoveries about the immune response. Two examples will serve to illustrate this pattern.

Alick Issacs and Jean Lindenmann, while working at the National Institute for Medical Research in London, found that addition of heat-inactivated influenza virus to the chorioallantoic membrane of chicken eggs interfered with the replication of influenza virus. When they published this observation in 1957, they coined the term "interferon".[88] In the 1970s, the protein was purified from cells by Sidney Pestka and Alan Waldman,[147] and subsequently the genes encoding the proteins were cloned.[71] This achievement allowed formal proof that IFN—by that time known to comprise a variety of different proteins—could interfere with viral replication. Extensive work with viruses showed that IFNs bind to cell surface receptors, and through the JAK-STAT signal transduction pathway, induce the synthesis of over 1000 mRNAs whose products establish an antiviral state in that cell.[38] Interferons protect against both viral and bacterial infections and also play a role in tumor clearance.

While working at the John Curtin School of Medical Research in Australia, Rolf Zinkernagel and Peter Doherty provided seminal insight on how cytotoxic T cells (CTLs) recognize virus-infected cells. They were studying mice infected with lymphocytic choriomeningitis virus (LCMV). Because this virus is noncytopathic, they hypothesized that brain damage in infected mice was a consequence of CTLs attacking virus-infected cells. They saw that CTLs isolated from LCMV-infected mice lysed virus-infected target cells *in vitro* only if both cell types had the same MHC haplotype. This requirement was termed MHC restriction.[194] In other words, a CTL must recognize two components on a virus-infected cell, one virus-specific, and one from the host. Subsequent research revealed that CTLs recognize a short viral peptide bound to MHC class I proteins on the surface of target cells. These observations revolutionized our understanding of T-cell mediated killing, establishing a foundation for understanding the general mechanisms used by the immune system to recognize both foreign microorganisms and self-molecules. The results have had wide implications for clinical medicine, not only in infection but also in areas such as cancer and autoimmune reactions in inflammatory diseases.

Emerging Viruses

In general, emerging viruses cause human infections that have not been seen or reported before (see Volume 1). They usually attract the public's attention, often by media sound bites like "killer viruses emerge from the jungle." The fact is that spread of infections through different hosts is well known in virology. Most so-called emerging infections represent zoonotic infections: infection of humans by a virus that normally exists in an animal population in nature.[172] Typically, these viruses have RNA genomes and some are spread to humans by insect vectors.

Perhaps the most infamous emerging virus infection of the 20th century is the human immunodeficiency virus, HIV-1, a retrovirus.[82] Progenitor HIV viruses exist in primates and we now believe they infected humans as a result of hunting and slaughter for food.[155] HIV was first recognized as a new disease entity by clinicians and epidemiologists in the early 1980s, and they rapidly tracked down the venereal mode of virus transmission. The virus was detected in blood products and transplant tissue. The immune system of HIV-infected individuals is severely compromised, which results in a variety of infections by usually benign microbes. The first published report of acquired immunodeficiency syndrome (AIDS) was in June 1981. Possible causative agents were first suggested in 1983[7] and then 1984.[66] Had this pandemic occurred in 1961 instead of 1981, neither the nature of retroviruses nor the existence of its host cell (CD-4 helper T-cell) would have been understood. HIV is a lentivirus (lenti is Latin for slow), and despite its recent appearance in humans, lentiviruses have been around for a long time. In fact, one of the first animal viruses to be identified in 1904 was the lentivirus that causes infectious equine anemia.

Many other examples of emerging viruses have attracted global concern and an exceptional rapid response of scientists and health officials.[172] The SARS and West Nile virus epidemics revealed the presence of a new human corona virus (SARS-CoV 1), identified with unprecedented speed, and the invasion of an Old World virus into the western hemisphere (West Nile virus).[86,131] In 2006, Chikungunya virus (an endemic virus infection in Africa) spread explosively to several countries where it was hitherto unknown.[154] On Reunion Island, more than 40% of the population of 800,000 people was infected. Other emerging infectious agents include Ebola virus, Lassa fever virus, Zika virus, and many mosquito-borne viruses. The first appearance of avian H5N1 virus in humans in 1999 produced fears of a pandemic of serious proportions since humans had no immunological history of infection by this avian strain.[167] Soon thereafter, the emergence of the pandemic H1N1 influenza virus in 2009 produced similar worries because of the relationship of the virus to the deadly 1918 influenza epidemic.[169] The mobilization of world health networks, public health officials, vaccine producers, veterinarians, clinicians, and molecular virologists marked a new chapter in dealing with emerging diseases. As the COVID-19 pandemic revealed, emerging viruses are and will be ongoing features of viral infections. There will

always be another potential pandemic in our future.

Epidemiology of Viral Infections

The study of the incidence, distribution, and control of disease in a population is an integral part of virology. The technology advancements of the last 50 years have provided epidemiology with a terrific boost. The discovery of specific molecular reagents (eg, recombinant DNA technology, antibodies, polymerase chain reaction [PCR], rapid diagnostic tests, high volume DNA and RNA sequencing) now enables detection of virions, proteins, and nucleic acids in body fluids, tissue samples, or in the environment. Moreover, we now can compare and classify viral isolates rapidly, determine the relationships between virus strains, and track the spread of infections around the world. The marriage of behavioral, geographic, and molecular epidemiology made this a most powerful science.[48]

The understanding of epidemics and pandemics of our most common viral infections such as influenza requires the perspective of ecology, population biology, and molecular biology.[100,167,169] G. Hirst and his colleagues (1941–1950) developed the diagnostic tools that permitted both the typing of the hemagglutinin (HA protein) of influenza A strains and the monitoring of the antibody response to this antigen in patients (see Chapters 42 and 43). These observations have been expanded, with more and more sophisticated molecular approaches, to prove the existence of animal reservoirs for influenza viruses, the reassortment of viral genome segments between human and animal virus strains (antigenic shift), and a high rate of mutation (antigenic drift) caused by RNA-dependent RNA synthesis with no known RNA editing or corrective mechanisms.[143] These molecular events that lead to episodic local epidemics and worldwide pandemics are understood in broad outline. Many viruses such as SARS CoV-2 are now known to evolve at high rates following basic darwinian principles in a time frame shorter than that of any other organism. Indeed, we now understand that RNA virus populations exist as a quasispecies or a swarm of individual viral genomes where every member is unique. Influenza viruses are successful because they have evolved to carry the very engines of evolution: mechanisms of mutation and recombination (reassortment). Influenza A virus has not been eliminated even with effective vaccines and antiviral drugs. Variants always arise that escape effective immune responses thorough high mutation (drift) and when coinfection occurs with viruses spreading from nonhuman hosts, new reassortants regularly arise. These new combinations of viral genes can change the pattern of infection from local to pandemic via an antigenic shift of its HA and NA subunit genes. These studies reveal an extraordinary lifestyle that reverberates around the planet in birds, farm animals, and humans. The study of the mechanisms of viral pathogenesis and modulation of the immune system has led to new insights in the virus-host relationship.

New technology discovered and developed over the past 35 years is changing the way viral infections are studied in the lab and in the field and is changing our appreciation of epidemiology and virus ecology.[166] Amplification technologies such as PCR permit rapid sampling of viral nucleic acids without growth in culture or plaque purification. Microarray technology where discriminatory DNA sequences from all sequenced viral genomes are put on a single array enables rapid classification of PCR amplified nucleic acids.[179] Rapid genome sequencing has revealed hitherto described viral genomes, relationships among viruses, and sequence heterogeneity within a virus population.[114] Mutations can be detected rapidly, documented, and localized in the viral genome. Importantly, the biological consequences can be monitored quickly. For example, in the late 1970s, viral epidemiologists were confronted with a highly transmissible, lethal infection of puppies.[134] In record time, scientists found that just two mutations in the capsid gene of feline parvovirus altered the host range such that the mutant could infect dogs. In less than a year, a completely new, highly pathogenic virus called canine parvovirus spread all around the world. Its evolution has continued to be monitored and a highly effective vaccine was developed. A similar type of molecular archeology enabled scientists to analyze serum samples collected from patients in the 1950s in efforts to understand the origins of HIV.[82] Sequence analysis of the HIV genome from one sample (ZR 1959) suggested that the virus may have emerged in the 1940s to 1950s. Field studies in Africa of viruses present in primate feces indicated that HIV most likely derived from a chimpanzee lentivirus in Africa.[155] After the initial human infection, rapid mutation and selection established the first human variants of this lentivirus that replicated and continued to evolve as they spread through their new human hosts.

We cannot forget the considerable impact of veterinary virus epidemiology on our understanding of complicated human diseases. For example, careful epidemiological work by Sigurdsson and colleagues on unusual diseases of sheep[160] provided the first understanding of slow infections in sheep (Visna-Maedi virus; a lentivirus) and infectious proteins (prions), which cause spongiform encephalopathies (Chapter 22).

As we will describe in the next section, molecular epidemiology is reaching new levels of sophistication, not only in detecting new viruses but also taking inventory of the viral ecosystem (the virome). Whether the next human epidemic will result from a novel variant of Ebola virus, coronavirus, or Norwalk virus or the more likely possibility of a new pandemic variant of influenza virus remains to be seen. The new technologies also enable analysis of virus populations in natural communities of nonhuman animals.[143] What is abundantly clear, however, is that the demographics of the human population on earth are changing at unprecedented rates (see Table 1.2). Even as birth rates slow, our planet will house 8-10 billion people by 2050–2100. For the first time, there will be three to four times more people above the age of 60 than below 3-4 years of age. Not only are we an aging population but we also are moving to urban environments with more than 20-30 cities containing more than 10 million people. Clearly, patterns of human behavior (increased population density, increased travel, increased ages of the population, income disparity, food shortages, and climate change) will provide the environment for the selection of emerging viruses and the challenges to the new field of molecular epidemiology.

TABLE 1.2 Advances and Challenges

Vaccines	Yellow fever virus vaccine, live attenuated Salk and Sabin vaccines for poliovirus, killed and live attenuated Recombinant hepatitis B vaccine, subunit Vaccinia virus vaccine to eradicate natural smallpox virus from the planet Influenza virus vaccines, inactivated and live attenuated Varicella-zoster virus vaccines, live attenuated Rotavirus vaccines, live attenuated Measles vaccines, live attenuated Recombinant human papillomavirus vaccine; subunit; prevents cancers and virus infections SARS CoV 2 mRNA vaccines, attenuated vaccines, adenovirus vectored vaccines, passive immunity with monoclonal antibodies
Antiviral Drugs	Acyclovir against herpes simplex type 1 and type 2 Combination therapy: protease, reverse transcriptase, and integrase inhibitors against HIV Interferon therapy for hepatitis B and C Amantadine against influenza virus A Neuraminidase inhibitors against influenza virus HCV replication inhibitors
Epidemiological advances	Understanding the molecular basis of antigenic shift and drift in influenza viruses Identification of the cause of AIDS and SARS Prion diseases recognized and mechanisms elucidated Deep sequencing, genome analysis, pathogen discovery, uncovering the molecular nature of epidemic and pandemic infections Recognition of the role of zoonotic infections in the emergence of new viral diseases Recognition of specific viruses as causative agents in human cancers Elucidation of the concept of viral quasispecies and the molecular biology of viral populations
Viral pathogenesis	Identification of viral virulence genes Identification of host genes affecting virus replication and spread Identification of the molecular bases for antiviral immune defenses (adaptive immunity) Identification of the molecular basis of front-line cellular defenses (intrinsic and innate immunity) including apoptosis and induction of defensive cytokines Understanding of the molecular basis for viral tropism Elucidation of the mechanisms involved in viral quiescence and persistence
The challenges (societal) The challenges (scientific)	Population explosion: more people now live on the planet than at any time in our existence (predicted to be 8-10 billion in the next few decades) Population concentration: world populations are concentrating in large urban centers of 10-20 million people or more Population demographics: for the first time, there are more people over the age of 60 than under the age of 4 Population interactions: world populations interact physically at rates and extents never before possible Acceptance of public health mitigation measures (social distancing, masks, vaccines, quarantine) Pandemic viral diseases and bioterrorism provide continuing challenges for human survival Research costs money: how do we alleviate the pressures on funding and support of fundamental research Discoveries cannot be predicted: how to balance true discovery research with applied (translational) research Public support: how do we develop support and advocacy for virology research and public health measures Policy makers must understand virology: more engagement of scientists with lawmakers and the general public Public education about vaccination and other public health issues Discovering an effective vaccine against HIV Developing vaccines against persistent viruses Discovering and developing new antiviral drugs Development of rapid viral diagnostic and identification strategies Coupling new technology with established procedures Balancing risks and benefits of dangerous pathogen research Developing surrogates for Koch's postulates in modern pathogen discovery programs Defining and understanding the composition and interplay of microbial communities inside and outside hosts (natural vs unnatural flora)

Host-Virus Interactions and Viral Pathogenesis

The technologies that contributed most to the modern era of virology (1960 to present) were advances in cell culture and molecular biology.[54] Virologists described the replicative cycles of viruses in great detail under well-defined conditions and demonstrated the elaborate interactions between viral genomes, viral proteins, and the host cellular machinery. As indicated previously, these advances resulted in an extraordinary inquiry into the functions of infected or uninfected host cells using the tools of both molecular and cell biology. As this approach matured, it became more reductionist in nature, and the questions became more detailed. However, some virologists used the new knowledge to move back to more complicated *in vivo* systems to study previously difficult problems in host-virus interactions involving the natural host or animal models of infection. Chief among these new questions was, how does a virus cause disease processes in the animal? How do we quantitate viral virulence and what is the genetic basis of an attenuated virus? How is a viral infection transmitted? These studies have identified, in selected viruses, a set of genes and functions that broadly impact our understanding of pathogenesis.

The COVID-19 Pandemic

(Section adapted from Ref.[40])

The world-wide spread of SARS-CoV-2 was devastating on a scale that few could have imagined. While there have been multiple viral pandemics in the last century, two stand out for their impact: the 1918 influenza pandemic and acquired immune deficiency syndrome (AIDS). Both killed millions of people and have had broad social and economic impacts. At this writing in January 2022, there are more than 400 million documented cases of COVID-19 worldwide and over 6 million deaths. Given the difficulties in testing massive numbers of people, the true prevalence of infection and death is undoubtedly much higher. The explosive spread of SARS-CoV-2 in late 2019 and early 2020 caught most of the world unprepared in many ways. Initially, there were few ways to control the spread except by old-fashioned quarantine methods: most of the world's population locked down, suspended travel, closed borders, and limited or stopped interactions of large groups of people. As a result, the medical disaster was compounded with social disruption and economic devastation.

The COVID-19 causative agent was isolated rapidly, identified as a coronavirus, and named SARS-CoV-2. While a new member of the *Coronaviridae*, it was not a "new" virus because coronaviruses are well known. Indeed, the first human coronavirus was described in 1964 by June Almeida in London. There are at least four known human coronaviruses that cause mild respiratory infections, including the common cold. Other animals can be infected with particular coronaviruses such as mouse hepatitis virus, infectious bronchitis virus of chickens, and transmissible gastroenteritis virus of swine. SARS-CoV-2 joins severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) coronaviruses, which emerged in 2002 and 2012, respectively, that cause severe human disease. SARS and MERS share some features with COVID-19 but each also has unique aspects. The SARS-CoV epidemic ended after ~8000 documented infections and that virus has not been since isolated from humans. MERS-CoV continues to infect several humans each month, mainly in the Arabian Peninsula.

Like most emerging viruses, SARS-CoV-2 most likely existed in an animal reservoir before it entered the human population. When and where SARS-CoV-2 entered humans has not been determined, although all evidence points to a bat origin: multiple SARS-CoV-2–related viruses have been identified from bats in China, Japan, Thailand, and Laos.

The genome sequence of some of the first virus isolates in China was widely disseminated to the research community. Within a few weeks, a global army of investigators was mobilized to study this new agent. This intense, scientific effort led to the rapid characterization of the SARS-CoV-2 attachment/fusion protein and the cell surface receptor was identified as the human enzyme ACE2, the same receptor used by SARS-CoV, the cause of SARS. Remarkably, the structure of the viral attachment protein (also called the spike protein) was determined at atomic-level resolution within a few months. Even more astounding was the development of new vaccines for production of the spike protein: mRNA vaccines, adenovirus vectored vaccines, and protein vaccines. Testing these vaccines for safety and efficacy was achieved faster than any other vaccine. The efficacy of these vaccines is nothing less than amazing. Nevertheless, the challenges of manufacturing, distributing, and public acceptance have been substantial.

Lessons Learned

(Section adapted from Ref.[40])

The most important lesson is that future pandemics are inevitable, and we must be ready for them. If we learned anything by being caught by surprise by the explosive spread of SARS-CoV-2, it is that rapid methods of surveillance and data sharing are essential. One challenge is how to recognize potential pandemics early and inform the public to allow prompt public health interventions. The world must have coordinated plans for action, to avoid confusion and inadequate responses. A second challenge is that we must identify and stockpile antiviral compounds and vaccines that can inhibit replication by a wide range of coronaviruses. Such therapeutics must have been tested for safety in humans so that at the first signs of a new outbreak they could be rapidly deployed.

We learned that transmission of infection can be slowed by physical distancing, wearing masks, washing hands, and staying home when ill. These efforts are classical public health procedures that we must not forget. An important lesson is that research in basic virology, cell biology, immunology, biochemistry, and other relevant areas is essential. These studies provide the necessary head start to meet new viruses before they overtake us and our health system, economy, and society. The fruits of such research enabled the rapid development of diagnostic tests, the use of DNA sequencing methods to characterize millions of genomes, and of course novel vaccines that are changing the face of the pandemic.

While these lessons are obvious, a more difficult challenge has emerged. COVID-19 has revealed deficiencies in how different societies operate and communicate as well as how politics can color public health responses. Since we live in a world of instant communication, dissemination of information, without regard to accuracy, is fast and seemingly uncontrollable. Moreover, the unwillingness of some institutional and political leaders as well as some members of the public to follow science-based guidelines and protocols is a serious problem that will continue long after the COVID-19 pandemic eases. More education is essential because much of the public and many of our public officials barely had any concept of viruses as infectious agents and their potential to upend our world. Until the public has some basic understanding of virology, history will repeat itself.

Past is Prolog

(Section adapted from Ref.[55])

The COVID-19 pandemic not only changed the world but also changed the study of virology as well. In general, virology has always been shaped by technology. When a virologist wants to answer a question, and current methods are inadequate, the virologist invents, borrows, or repurposes to develop a new technique. Many techniques and methods deployed to study SARS-CoV-2 and COVID-19 were not available just a few years ago. Moreover, the integration of new technology with public health measures to detect viral genomes and proteins as well as the immune response to infections has been revolutionary. New vaccine technology has enabled discovery, production, and mobilization of vaccines with remarkable efficacy.

Deep, high-throughput sequencing, the relatively unbiased methods to determine the entire genome sequence of viruses both known and unknown, has revolutionized virology because it enables characterization of millions of genomes in a short time. Molecular epidemiology, learning about the transmission of infections in real time, and the study of virus evolution have been changed forever by this new technology. At the molecular level, structural biology has been changed dramatically by cryoelectron microscopy. The atomic structure of individual viral proteins or intact virus particles, which used to take many years dominated by trial and error, can be determined in less than a month. Artificial

intelligence programs have revolutionized the prediction of protein structures. Antiviral drug discoveries that relied on painstaking work to identify potential molecular targets followed by tedious screens are relics of the past. With new genetics technology, imaging, and powerful chemistry methods, potential cellular targets for antivirals can be identified in weeks.

The COVID-19 pandemic mobilized powerful technology leading to rapid applied and fundamental progress. The drive to develop therapeutics and vaccines as well as to discover how viruses work was remarkable and productive. The testing and Food and Drug Administration emergency use authorization of two vaccines within less than a year of the first genome sequence being posted—and more remarkably, both based on a vaccine platform that had not been previously used at any scale in humans for vaccine purposes—is without precedent. The kinetics and scale of molecular epidemiology is remarkable. With millions of SARS-CoV-2 genome sequences, we know almost as much about the evolution of this virus as any other.

Fortunately, the pace of work and depth of analysis are applicable to many viruses, not just coronaviruses or the next pandemic virus. These technological advances move all of biology forward. In the midst of this worldwide calamity, the new revolution in virology brings hope for the future. There will be another pandemic, and lessons learned from this one will help us with the next.

The Future of Virology

The future of virology is unpredictable but is guaranteed to be exciting. Who knows what discoveries remain? Certainly, the number of astounding and groundbreaking developments in biology over the past 60 years is remarkable.[54] Most could not have been predicted or even imagined, prior to their discovery. That virologists participated in making many of these discoveries is no accident: viral gene products have evolved to engage all the key nodes of biology ranging from the atomic to the organismal. We only must be smart enough to figure out how to identify these nodes. The forces that will drive our field are technology development, public health, information processing, and, of course, personal curiosity. Indeed, new life science technologies invariably will give rise to new, unexpected insights in virology to meet our current challenges. That has been and continues to be the future of virology (see Table 1.2).

A Role for Systems Biology in Virology

Not too long ago, molecular virology was limited to studies of one virus and one gene or gene product at a time. More complex studies often were seen as “descriptive.” Times have changed! New technology enables virologists to interrogate simultaneously many viruses and large groups of genes or gene products in ever expanding environments and biological networks. In this context, a network is defined as the interconnected intracellular processes that control everything within a cell: for example, DNA replication, processes of gene expression, organelle biogenesis, and metabolism to name a few.[130] The definition also encompasses networks of intercellular communication at the tissue, organ, and whole-organism level. Virologists are beginning to embrace a tenet of systems biology where information flows through these networks and disease arises when these networks are perturbed. Viral gene products cause changes in network architecture and thereby alter the dynamics of information flow. Future studies of viral pathogenesis are likely to involve identification and understanding of specific viral signatures of network imbalance that do not affect just one pathway but alter the fundamental homeostatic balance.[19,54,141,164]

Genomics and the Predictive Power of Sequence Analysis

The development of technological advances in biology often drives new approaches and permits one to ask novel questions that could not even be framed in the past. In the last decade of the 20th century, rapid and inexpensive DNA sequencing methods opened the way to sequence the genomes of many viruses and their hosts. This created large databases containing information about the variation of DNA or RNA sequences within a single virus (eg, HIV, influenza, coronaviruses) and permitted predictions about the nature of the mutations driving selective changes, mutation frequencies of different viruses, and evolutionary changes from isolates around the world. The correlations of these sequence variations with drug and vaccine resistance, changes in the genetic background of the host, and virulence have been informative.

Host genomes contain an amazing number of viral or viral-related sequences.[11,12] More than 50% of the DNA sequences found in the human genome were derived from retroviruses, retrotransposons, DNA transposons and randomly amplified sequences of genes (SINES and the 7S RNA gene), pseudogenes, and repetitive DNA sequences.[106,177] Viruses certainly have left a major mark upon the evolution of their host’s genomes in addition to the selective pressures they exert via virus infections and deaths. During the evolution of humans from their ancestral line, retroviruses and retrotransposons (the LINE-1 elements) have entered the germ line, amplified their copy numbers, and integrated at various sites in the genome. This process introduces mutations, alters patterns of gene expression, and creates new interactions of viruses with their hosts. This is clearly one of the drivers of host evolution. Over time, these retroviruses (human endogenous retroviruses or HERVs) accumulate mutations in their genes, and some recombine out of the genome leaving only the LTRs as a remnant marking their past insertion. While humans no longer contain viable HERVs, the multiple copies of HERV-H or HERV-K viruses when transcribed in cells produce functional viral proteins from different copies of these viruses, and the viral particles that are produced are defective and very poorly transmitted. Cellular transcription factors regulate the expression of the HERVs, and the p53 transcription factor, activated by stress and DNA damage, transcribes the HERV H genome and produces particles in response to such stress.[192] Similarly, the LINE-1 retrotransposons, which have about 300 viable and movable elements in the human genome today, are responsible for about 1% of the mutations found in each generation. Line-1 transposons also contain p53 DNA response elements[83] and are also regulated by stress responses recorded by the host. While it is clear that retroviruses and transposons can shape the host genome, it is equally clear that the host genome is a place for new viral genomes to evolve, recombine with exogenous viral genomes and possibly produce a new agent optimized for replication in its host. Understanding the dynamics of these vestiges of viruses that reside in our genome is a challenge for the future.

With many host genome sequences representing all kingdoms of life in the databases, it has been possible to do some rather eye-opening analyses. For example, the resurrection of endogenous retroviruses from inactive sequences in host DNA has allowed the investigation of interactions between extinct pathogens called paleoviruses and their hosts that occurred millions of years ago.[52] By cloning these sequences, it has been possible to identify the cellular receptor of these extinct retroviruses.[157] Perhaps more amazing is that similar “viral genome fossils” representing DNA copies of filoviruses and bornaviruses as well as parvoviruses and circoviruses have been found in a variety of host genomes.[11,12] When the evolutionary history of various host genomes harboring these viral sequences were compared, it was possible to deduce that ancestors of modern viruses were in existence millions of years ago. What is even more curious is that these genome insertion events seemed to happen around the same time in a wide variety of mammals. What global event could have stimulated such activities?

The Virome: How Many Viruses Are There and Where Are They?

Virus ecology, as a result of modern virus discovery technology, is posing many questions.[100,166] In 1977, when Fred Sanger sequenced the DNA genome of coliphage phiX174, many virologists were impressed with the wealth of information contained in a "simple" DNA sequence and the congruence of genetic and biochemical data with the genome structure. The recent discovery of "giant viruses" that infect amoeba revealed that the time-honored definition of viruses as small filterable agents no longer holds (Chapter 17).

In <25 years, sampling, sequencing, and computer technology now provide the wherewithal to identify and sequence entire viral communities from their natural environment without the intervention of time-held techniques of isolation and characterization of individual viruses.[46,162,179] In early 2003, a novel viral DNA microarray was used to reveal and partially sequence a previously uncharacterized coronavirus in a viral isolate cultured from a SARS patient. This chip technology has advanced to the point that essentially all the known viral genomes can be represented on a single microarray. The technology may be obsolete already. The SARS-CoV-2 virus was identified quickly by high throughput sequencing technology. New techniques for discovery and analysis of viral populations are certain to be found. As can be expected in this "omics" era, the identification and study of an entire community of viruses in their natural habitat has been called metagenomics.[2,145,186] The diversity of viruses in the environment was unknown as we were limited to studying only those viruses that are easy to work with in the laboratory or those that have major impact on human health. The first metagenomic studies on viruses have revealed stunning diversity of genes and gene products that remain to be understood even in principle.[162,187] The combination of host and bacteriophage genome sequencing in the bacteria has proven to be an exceptional window on genome evolution and gene transfer. The practical value of identifying new gene products with novel functions cannot be overestimated. The repertoire of tactics for gene control and regulation is far more extensive than any of us imagined before the era of metagenomics. We can only expect that as the metagenomics of animal and plant viruses advances, the impact of knowing everything that is out there and the resulting knowledge of the dynamics of host-parasite interactions will be mind-boggling.[163]

Pathogen Discovery

With the plethora of viruses found in and on every living thing, defining new pathogens is challenging. Perhaps of fundamental importance is that proof of causation can no longer rely on the time honored Koch's postulates.[87] This assertion is made not only because it may be difficult to propagate new viruses and find models to test their pathogenicity but it also is likely that many diseases will involve the interaction of multiple microbial communities (viruses, bacteria, fungi) that will be difficult to reproduce in the laboratory. Pathogen discovery will require new biomarkers of health and disease, methods to improve sampling and stability of samples, and technology to record relevant data and capacity to associate all these data with the sample. In the past, pathogen identification methods were slow and tedious and working with multiple samples was difficult if not impossible. It is now possible to collect and analyze serial samples over time as patients move from health to disease. Assembling data, maintaining data bases, and providing access for analysis will also involve advances in software and bioinformatics. In the end, the fundamental challenge will be how one moves from correlation of the presence of an agent or agents in disease to proof of causation.

Whether or not viruses are living has been debated since their discovery. The virus particle is certainly not alive as it is a collection of chemicals that is inert unless it enters a cell to deliver its nucleic acid genome. However, the infected cell displays all the qualities of life, including energy generation and evolution. Hence, when considering this question, we must distinguish between the virus particle and the virus-infected cell.

The idea that the virus particle and the infected cell are distinct was first proposed by Bandea in 1983.[6] He suggested that a virus is an organism without a cohesive morphological structure, with subsystems that are not in structural continuity:

Viruses are presented as organisms which pass in their ontogenetic cycle through two distinctive phenotypic phases: (1) the vegetative phase and (2) the phase of viral particle or nucleic acid. In the vegetative phase, considered herein to be the ontogenetically mature phase of viruses, their component molecules are dispersed within the host cell. In this phase, the virus shows the major physiological properties of other organisms: metabolism, growth, and reproduction.

According to Bandea's hypothesis, the infected cell is the virus, while the virus particles are "spores" or reproductive forms. His theory was largely ignored until the discovery of the giant mimivirus: its DNA genome replicates and new virions are produced in the cytoplasm within complex viral "factories." Claverie suggested that the viral factory corresponds to the organism, whereas the virus particle is used to spread from cell to cell. He wrote that "to confuse the virion with the virus would be the same as to confuse a sperm cell with a human being".[29]

If we accept that the virus is the infected cell, then it becomes clear that most virologists have confused the virus particle and the virus. This situation is probably a consequence of the fact that modern virology is rooted in the study of bacteriophages that began in the 1940s. These viruses were not found to induce cellular factories and disappear (the eclipse phase) early after cell entry. Contemporary examples of such confusion include the production by structural virologists of virus crystals, and the observation that viruses are the most abundant entities in the seas. In both cases, it is the virus particle that is being studied. But virologists are not the only ones at fault—the media writes about the AIDS virus or COVID-19 while showing illustrations of the virus particle.

Forterre[60] writes that those who consider the virus to be the infected cell also believe that viruses are alive:

...one can conclude that infected eukaryotic cells in which viral factories have taken control of the cellular machinery became viruses themselves, the viral factory being in that case the equivalent of the nucleus. By adopting this viewpoint, one should finally consider viruses as cellular organisms. They are of course a particular form of cellular organism, since they do not encode their own ribosomes and cell membranes but borrow those from the cells in which they live.

This argument leads to the assumption that viruses—not virus particles—are living, according to the classical definition of living organisms as cellular organisms. In other words, a virus is an organism with two phases, the virus particle (which is not living) and the infected cell (which is living).

REFERENCES

1. Alberts BM, Bedinger BP, Formosa T. Studies on DNA replication in the bacteriophage T4 in vitro systems. *Cold Spring Harbor Symp Quant*

Biol. 1982:655-668.

2. Angly E, Felts B, Breitbart M, et al. The marine viromes of four oceanic regions. PLoS Biol. 2006;4(11):e368.

3. Arvin AM, Greenberg HB. New viral vaccines. Virology. 2006;344(1):240-249.

4. Astrachan L, Volkin E. Properties of ribonucleic acid turnover in T2-infected Escherichia coli. Biochim Biophys Acta. 1958;29(3):536-544.

5. Baltimore D. RNA-dependent DNA polymerase in virions of RNA tumour viruses. Nature. 1970;226(5252):1209-1211.

6. Bandea CI. A new theory on the origin and the nature of viruses. J Theor Biol. 1983;105(4):591-602.

7. Barre-Sinoussi F, Chermann JC, Rey F, et al. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science. 1983;220(4599):868-871.

8. Bawden FC. Liquid crystalline substances from virus infected plants. *Nature*. 1936;138:1051-1052.

9. Bawden FC, Pirie NW. The isolation and some properties of liquid crystalline substances from solanaceous plants infected with three strains of tobacco mosaic virus. *Proc R Soc Med*. 1937;123:274-320.

10. Beijerinck M. Concerning a contagium vivum fluidum as a cause of the spot-disease of tobacco leaves. *Verh Akad Wetensch*. 1898;6:3-21.

11. Belyi VA, Levine AJ, Skalka AM. Sequences from ancestral single-stranded DNA viruses in vertebrate genomes: the parvoviridae and circoviridae are more than 40 to 50 million years old. J Virol. 2010;84(23):12458-12462.

12. Belyi VA, Levine AJ, Skalka AM. Unexpected inheritance: multiple integrations of ancient bornavirus and ebolavirus/marburgvirus sequences in vertebrate genomes. PLoS Pathog. 2010;6(7):e1001030.

13. Benzer S. Fine structure of a genetic region in bacteriophage. Proc Natl Acad Sci U S A. 1955;41(6):344-354.

14. Benzer S, Champe SP. Ambivalent rII mutants of phage T4. Proc Natl Acad Sci U S A. 1961;47(7):1025-1038.

15. Berget SM, Moore C, Sharp PA. Spliced segments at the 5' terminus of adenovirus 2 late mRNA. Proc Natl Acad Sci U S A. 1977;74(8):3171-3175.

16. Bernal JD, Fankuchen I. X-ray and crystallographic studies of plant virus preparations. *J Gen Physiol*. 1941;25(1):147-165.

17. Bernal JD, Fankuchen I. X-ray and crystallographic studies of plant virus preparations: I. Introduction and preparation of specimens II. Modes of aggregation of the virus particles. J Gen Physiol. 1941;25(1):111-146.

18. Biggs PM, Payne LN, Milne BS, et al. Field trials with an attenuated cell associated vaccine for Marek's disease. Vet Rec. 1970;87(23):704-709.

19. Birungi G, Chen SM, Loy BP, Ng ML, Li SF. Metabolomics approach for investigation of effects of dengue virus infection using the EA.hy926 cell line. J Proteome Res. 2010;9(12):6523-6534.

20. Blumberg BS, Gerstley BJ, Hungerford DA, London WT, Sutnick AI. A serum antigen (Australia antigen) in Down's syndrome, leukemia, and hepatitis. Ann Intern Med. 1967;66(5):924-931.

21. Bohmann D, Bos TJ, Admon A, Nishimura T, Vogt PK, Tjian R. Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science. 1987;238(4832):1386-1392.

22. Bordet J. Concerning the theories of the so-called "bacteriophage". *Br Med J*. 1922;2:296.

23. Buonaguro L, Pulendran B. Immunogenomics and systems biology of vaccines. Immunol Rev. 2011;239(1):197-208.

24. Burkitt D. A children's cancer dependent on climatic factors. *Nature*. 1962;194:232-234.

25. Butler PJ, Klug A. Assembly of the particle of tobacco mosaic virus from RNA and disks of protein. *Nat New Biol*. 1971;229:47-50.

26. Campbell AM. Episomes. *Adv Genet*. 1962;11:101-145.

27. Choo QL, Quo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science. 1989;244(4902):359-362.

28. Chow LT, Gelinas RE, Broker TR, Roberts RJ. An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. Cell. 1977;12(1):1-8.

29. Claverie JM. Viruses take center stage in cellular evolution. Genome Biol. 2006;7(6):110.

31. Cohen SS. The synthesis of bacterial viruses; the synthesis of nucleic acid and protein in Escherichia coli B infected with T2r + bacteriophage. J Biol Chem. 1948;174:281-293.

32. Cohen SS. Virus-induced Enzymes. Columbia University Press; 1953.

33. Crick FH, Watson JD. Structure of small viruses. *Nature*. 1956;177:473-475.

34. D'Herelle F. Sur un microbe invisible antagoniste des bacilles dysentériques. *CR Hebd Seances Acad Sci Paris*. 1917;1:72-99.

35. D'Herelle F. Le microbe bactériophage, agent d'immunité dans la peste et le barbone. *C R Seances Acad Sci III*. 1921;72:99.

36. D'Herelle F. The Bacteriophage and Its Behavior. Williams & Wilkins; 1926.

37. Danna K, Nathans D. Specific cleavage of simian virus 40 DNA by restriction endonuclease of Hemophilus influenzae. Proc Natl Acad Sci U S A. 1971;68(12):2913-2917.

38. Danna KJ, Sack Jr GH, Nathans D. Studies of simian virus 40 DNA. VII. A cleavage map of the SV40 genome. J Mol Biol. 1973;78(2):363-376.

39. Darnell JE Jr, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science. 1994;264(5164):1415-1421.

40. DeCaprio JA, Ludlow JW, Figge J, et al. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell. 1988;54(2): 275-283.

41. DiMaio D, Enquist LW, Dermody TS. A new coronavirus emerges, this time causing a pandemic. Annu Rev Virol. 2020;7(1):iii-v.

42. Dulbecco R, Vogt M. Some problems of animal virology as studied by the plaque technique. *Cold Spring Harb Symp Quant Biol*. 1953;18:273-279.

43. Dulbecco R, Vogt M. Evidence for a ring structure of polyoma virus DNA. *Proc Natl Acad Sci U S A*. 1963;50:236-243.

44. Dynan WS, Tjian R. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell. 1983;35(1):79-87.

45. Eagle H. The specific amino acid requirements of a human carcinoma cell (Stain HeLa) in tissue culture. J Exp Med. 1955;102(1):37-48.

46. Edgar RS, Wood WB. Morphogenesis of bacteriophage T4 in extracts of mutant-infected cells. *Proc Natl Acad Sci U S A*. 1966;55:498-505.

47. Edwards RA, Rohwer F. Viral metagenomics. *Nat Rev Microbiol*. 2005;3(6):504-510.

48. Eidson CS, Kleven S. Anderson DP. Vaccination Against Marek's Disease. Oncogenesis and Herpesvirus; 1972.

49. Elena S, Bedhomme S, Carrasco P, et al. The evolutionary genetics of emerging plant RNA viruses. Mol Plant Microbe Interact. 2011;24(3):287-293.

50. Ellerman V, Bang O. Experimentelle Leukamie bei Huhnern. Zentralbl Bakteriol 1 Orig. 1908;46:595-597.

51. Ellis EL. Bacteriophage: one-step growth. In: Carirns J, Stent G, Watson JD, eds. Phage and the Origins of Molecular Biology. Cold Spring Harbor Laboratory Press; 1966.

52. Ellis EL, Delbruck M. The growth of bacteriophage. *J Gen Physiol*. 1939;22:365-384.

53. Emerman M, Malik HS. Paleovirology--modern consequences of ancient viruses. *PLoS Biol*. 2010;(2):e1000301.

54. Enders JF, Weller TH, Robbins FC. Cultivation of the lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. Science. 1949;109(2822):85-87.

55. Enquist LW; Editors of the Journal of Virology. Virology in the 21st century. J Virol. 2009;83(11):5296-5308.

56. Enquist LW, DiMaio D, Dermody TS. Recurring revolutions in virology. Annu Rev Virol. 2021;8(1):v-vii.

57. Epstein MA, Achong BG, Barr YM. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet. 1964;1(7335):702-703.

58. Fenner F, Nakano JJ. Poxviridae: the poxviruses. In: Lennette EH, Halonen P, Murphy FA, eds. The Laboratory Diagnosis of Infectious Diseases: Principles and Practice, Viral, Rickettsial, and Chlamydial Diseases. Springer-Verlag; 1988.

59. Fitzgerald M, Shenk T. The sequence 5'-AAUAAA-3'forms parts of the recognition site for polyadenylation of late SV40 mRNAs. Cell. 1981;24(1):251-260.

60. Flaks JG, Cohen SS. Virus-induced acquisition of metabolic function. I. Enzymatic formation of 5-hydroxymethyldeoxycytidylate. J Biol Chem. 1959;234:1501-1506.

61. Forterre P. Defining life: the virus viewpoint. Orig Life Evol Biosph. 2010;40(2):151-160.

62. Fowlkes DM, Shenk T. Transcriptional control regions of the adenovirus VAI RNA gene. Cell. 1980;22(2 Pt 2):405-413.

63. Fraenkel-Conrat H, Singer B. The chemical basis for the mutagenicity of hydroxylamine and methoxyamine. *Biochim Biophys Acta*. 1972;262:264-268.

64. Fraenkel-Conrat H, Singer BJ, Williams RC. Infectivity of viral nucleic acid. *Biochim Biophys Acta*. 1957;25:87-96.

65. Furth J, Strumia M. Studies on transmissible lymphoid leucemia of mice. J Exp Med. 1931;53(5):715-731.

66. Furuichi Y, Morgan M, Muthukrishnan S, Shatkin AJ. Reovirus messenger RNA contains a methylated, blocked 5'-terminal structure: m-7G(5')ppp(5')G-MpCp. Proc Natl Acad Sci U S A. 1975;72(1):362-366.

67. Gallo RC, Salahuddin SZ, Popovic M, et al. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS

and at risk for AIDS. Science. 1984;224(4648):500-503.

68. Gaynor RB, Hillman D, Berk AJ. Adenovirus early region 1A protein activates transcription of a nonviral gene introduced into mammalian cells by infection or transfection. Proc Natl Acad Sci U S A. 1984;81(4):1193-1197.

69. Germond JE, Hirt B, Oudet P, Chambon P. Folding of the DNA double helix in chromatin-like structures from simian virus 40. Proc Natl Acad Sci U S A. 1975;72(5):1843-1847.

70. Gey GO, Coffman WD, Kubicek MT. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res*. 1952;12:264-265.

71. Girer A, Schramm G. Infectivity of ribonucleic acid from tobacco mosaic virus. *Nature*. 1956;177:702-703.

72. Goeddel DV, Shepard HM, Yelverton E, et al. Synthesis of human fibroblast interferon by E. coli. Nucleic Acids Res. 1980;8(18):4057-4074.

73. Goff SP, Berg P. Construction of hybrid viruses containing SV40 and lambda phage DNA segments and their propagation in cultured monkey cells. Cell. 1976;9(4 PT 2):695-705.

74. Gribskov M, Burgess RR. Sigma factors from E. coli, B. subtilis, phage SP01, and phage T4 are homologous proteins. Nucleic Acids Res. 1986;14(16): 6745-6763.

75. Gruss P, Dhar R, Khoury G. Simian virus 40 tandem repeated sequences as an element of the early promoter. *Proc Natl Acad Sci U S A*. 1981;78:943-947.

76. Hayward WS, Neel BG, Astrin SM. Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature. 1981;290(5806):475-480.

77. Henderson DA. Principles and lessons from the smallpox eradication programme. Bull World Health Organ. 1987;65(4):535-546.

78. Henderson DA. Smallpox virus destruction and the implications of a new vaccine. Biosecur Bioterror. 2011;9(2):163-168.

79. Henle G, Henle W. Diehl V. Relation of Burkitt's tumor-associated herpes-ytpe virus to infectious mononucleosis. Proc Natl Acad Sci U S A. 1968;59(1):94-101.

80. Hershey AD. The Bacteriophage Lambda. Cold Spring Harbor Laboratory Press; 1971.

81. Hershey AD, Chase M. Independent functions of viral protein and nucleic acid in growth of bacteriophage. *J Gen Physiol*. 1952;36:39-56.

82. Hilleman MR. Historical and contemporary perspectives in vaccine developments: from the vantage of cancer. *Prog Med Virol*. 1992;39:1-18.

83. Ho DD, Bieniasz PD. HIV-1 at 25. Cell. 2008;133(4):561-565.

84. Hoh J, Jin S, Parrado T, Edington J, Levine AJ, Ott J. The p53MH algorithm and its application in detecting p53-responsive genes. *Proc Natl Acad Sci U S A*. 2002;99(13):8467-8472.

85. Holmes FA. Local lesions in tobacco mosaic. *Bot Gaz*. 1929;87:39-55.

86. Hughes SS. The Virus: A History of the Concept. Heinemann Education Books; 1977.

87. Hui DS, Chan PK. Severe acute respiratory syndrome and coronavirus. Infect Dis Clin North Am. 2010;24(3):619-638.

88. Inglis TJJ. Principia aetiologica: taking causality beyond Koch's postulates. J Med Microbiol. 2007;56(Pt 11):1419-1422.

89. Isaacs A, Lindenmann J. Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci. 1957;147(927):258-267.

90. Isaacs A, Lindenmann J, Valentine RC. Virus interference. II. Some properties of interferon. Proc R Soc Lond B Biol Sci. 1957;147(927):268-273.

91. Ivanofsky D. Concerning the mosaic disease of the tobacco plant. *St. Petersburg Acad Imp Sci Bull*. 1892;35:67-70.

92. Ivanofsky D. On the mosaic disease of tobacco. *Zeitschrift fur Pfanzenkrankheit*. 1903;13:1-41.

93. Jackson DA, Symons RH, Berg P. Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of Escherichia coli. Proc Natl Acad Sci U S A. 1972;69(10):2904-2909.

94. Jacob F, Wollman E. Etude génétique d'un bactériophage tempéré d'Escherichia coli. I. Le système génétique du bactériophage l. Ann Inst Pasteur. 1954;87:653-673.

95. Jacob F, Wollman E. Sexuality and the Genetics of Bacteria. Academic Press; 1961.

96. Jang SK, Davies MV, Kaufman RJ, Wimmer E. Initiation of protein synthesis by internal entry of ribosomes into the 5' nontranslated region of encephalomyocarditis virus RNA in vivo. J Virol. 1989;63(4):1651-1660.

97. Jensen JH. Isolation of yellow-mosaic virus from plants infected with tobacco mosaic. *Phytopathology*. 1933;23:964-974.

98. Kates J, Beeson J. Ribonucleic acid synthesis in vaccinia virus. II. Synthesis of polyriboadenylic acid. J Mol Biol. 1970;50(1):19-33.

99. Kates JR, McAuslan BR. Poxvirus DNA-dependent RNA polymerase. Proc Natl Acad Sci U S A. 1967;58(1):134-141.

100. Kausche G, Ankuch PF, Ruska H. Die Sichtbarmachung von pflanzlichem Virus in Ubermikroskop. *Naturwissenschaften*. 1939;27:292-299.

101. Keesing F, Belden LK, Daszak P, et al. Impacts of biodiversity on the emergence and transmission of infectious diseases. Nature. 2010;468(7324):647-652.

102. Kitajewski J, Schneider RJ, Safer B, Shenk T. An adenovirus mutant unable to express VAI RNA displays different growth responses and sensitivity to interferon in various host cell lines. Mol Cell Biol. 1986;6(12):4493-4498.

103. Kornberg A. Biologic synthesis of deoxyribonucleic acid. *Science*. 1960;131:1503-1508.

104. Krugman S, Giles JP, Hammond J. Infectious hepatitis. Evidence for two distinctive clinical, epidemiological, and immunological types of infection. JAMA. 1967;200(5):365-373.

105. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227(5259):680-685.

106. Laemmli U, Cheng SM, Adolph KW, Paulson JR, Brown JA, Baymbach WR. Metaphase chromosome structure: the role of nonhistone proteins. Cold Spring Harb Symp Quant Biol. 1978;42(Pt 1):351-360.

107. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. Nature. 2001;409(6822):860-921.

108. Lane DP, Crawford LV. T antigen is bound to a host protein in SV40-transformed cells. Nature. 1979;278(5701):261-263.

109. Lee W. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. *Nature*. 1987;325:368-372.

110. Levine AJ. The tumor suppressor genes. *Annu Rev Biochem*. 1993;62:623-651.

111. Levine AJ. The origins of the small DNA tumor viruses. *Adv Cancer Res*. 1994;65:141-168.

112. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. Nature. 1991;351(6326):453-456.

113. Linzer DI, Levine AJ. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell. 1979;17(1):43-52.

114. Loeffler F, Frosch P. Zentralbl Bakteriol 1 Orig. 1901;28:371.

115. Long CD, Turner-Shelef K, Relman DA. Building a better virus trap. Trends Biotechnol. 2007;25(12):535-538.

116. Luria S. Mutations of bacterial viruses affecting their host range. *Genetics*. 1945;30:84-99.

117. Luria S. General Virology. Wiley; 1953.

118. Luria S, Anderson TF. The identification and characterization of bacteriophages with the electron microscope. *Proc Natl Acad Sci U S A*. 1942;28:127-130.

119. Luria SE, Darnell JE. General Virology. Wiley and Sons; 1967.

120. Lwoff A. The Prophage and I: Phage and the Origins of Molecular Biology. Cold Spring Harbor Press; 1961.

121. Lwoff A, Siminovitch L, Kjeldgaard N. Induction de la lyse bactériophagique de la totalité d'une population microbienne lysogène. *C R Seances Acad Sci III*. 1950;231:190-191.

122. Mayer A. On the mosaic disease of tobacco: preliminary communication. *Tijdschr Landbouwk*. 1882;2:359-364.

123. Mayer A. On the mosaic disease of tobacco. *Landwn VerSStnen*. 1886;32:451-467.

124. McKinney HH. Factors affecting the properties of a virus. *Phytopathology*. 1926;16:753-758.

125. McKinney HH. Mosaic diseases in the Canary islands. *J Agric Res*. 1929;39:557-578.

126. Monod J, Wollman E. L'inhibition de la croissance et de l'adaption enzymatique chez les bactéries infectées par le bactériophage. *Ann Inst Pasteur*. 1947;73:937-957.

127. Moore PS, Chang Y. Why do viruses cause cancer? Highlights of the first century of human tumour virology. Nat Rev Cancer. 2010;10(12):878-889.

128. Morrow JF, Berg P. Cleavage of Simian virus 40 DNA at a unique site by a bacterial restriction enzyme. Proc Natl Acad Sci U S A. 1972;69(11):3365-3369.

129. Morse ML, Lederberg EM, Lederberg J. Transduction in Escherichia coli K-12. Genetics. 1956;41(1):142-156.

130. Mulder C, Delius H. Specificity of the break produced by restricting endonuclease R 1 in Simian virus 40 DNA, as revealed by partial denaturation mapping. Proc Natl Acad Sci U S A. 1972;69(11):3215-3219.

131. Munger J, Bennett BD, Parikh A, et al. Systems-level metabolic flux profiling identifies fatty acid synthesis as a target for antiviral therapy. Nat Biotechnol. 2008;26(10):1179-1186.

132. Murray KO, Mertens E, Despres P. West Nile virus and its emergence in the United States of America. Vet Res. 2010;41(6):67.

133. Nathanson N, Kew OM. From emergence to eradication: the epidemiology of poliomyelitis deconstructed. Am J Epidemiol. 2010;172(11):1213-1229.

134. Negri A. Beitrag zum Stadium der Aetiologie der Tollwuth. *Z Hyg Infektionskrankh*. 1903;43:507-528.

135. Parrish CR, Kawaoka Y. The origins of new pandemic viruses: the acquisition of new host ranges by canine parvovirus and influenza A viruses. *Annu Rev Microbiol*. 2005;59:553-586.

136. Pasteur L. Méthode pour prévenir la rage apres morsure. *CR Acad Sci*. 1885;101:765-772.

137. Pelletier J, Sonenberg N. Internal binding of eucaryotic ribosomes on poliovirus RNA: translation in HeLa cell extracts. J Virol. 1989;63(1):441-444.

138. Pilder S, Moore M, Logan J, Shenk T. The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. Mol Cell Biol. 1986;6(2):470-476.

139. Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc Natl Acad Sci U S A. 1980;77(12):7415-7419.

140. Ptashne M. A Genetic Switch, Gene Control and Phage Lambda. Blackwell Science; 1987.

141. Purdy-Beale HA. Immunologic reactions with tobacco mosaic virus. *J Exp Med*. 1929;49:919-935.

142. Qian X, Yoon BJ. Comparative analysis of protein interaction networks reveals that conserved pathways are susceptible to HIV-1 interception. BMC Bioinformatics. 2011;12(Suppl 1):S19.

143. Racaniello VR, Baltimore D. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science. 1981;214(4523):916-919.

144. Rambaut A, Pybus OG, Nelson MI, Viboud C, Taubenberger JK, Holmes EC. The genomic and epidemiological dynamics of human influenza A virus. Nature. 2008;453(7195):615-619.

145. Reed W. Senate Documents. 1901:156.

146. Reyes A, Haynes M, Hanson N, et al. Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature. 2010;466(7304):334-338.

147. Rous P. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. *J Exp Med*. 1911;13:397-411.

148. Rubinstein M, Rubinstein S, Familleti PC, et al. Human leukocyte interferon purified to homogeneity. Science. 1978;202(4374):1289-1290.

149. Samulski RJ, Chang LS, Shenk T. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. J Virol. 1989;63(9):3822-3828.

150. Sanford KK, Earle WR, Likely GD. The growth in vitro of single isolated tissue cells. J Natl Cancer Inst. 1948;9(3):229-246.

151. Sarnow P, Ho YS, Williams J, Levine AJ. Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. Cell. 1982;28(2):387-394.

152. Schlesinger M. Die Bestimmung von Teilchengrïsse und Spezifischem gewicht des Bakteriophagen durch Zentrifugierversuche. *Z Hyg Infektionskrankh*. 1932;114:161.

153. Schlesinger M. Zur Frage der chemischen Zusammensetzung des Bakteriophagen. *Biochem Z*. 1934;273:306-311.

154. Schultz-Cherry S, Jones JC. Influenza vaccines: the good, the bad, and the eggs. *Adv Virus Res*. 2010;77:63-84.

155. Schwartz O, Albert ML. Biology and pathogenesis of chikungunya virus. Nat Rev Microbiol. 2010;8(7):491-500.

156. Sharp M, Hahn BH. The evolution of HIV-1 and the origin of AIDS. Philos Trans R Soc Lond B Biol Sci. 2010;365(1552):2487-2494.

157. Sinsheimer RL. A single-stranded DNA from bacteriophage phi X174. *Brookhaven Symp Biol*. 1959;12:27-34.

158. Soll SJ, Neil SJ, Bieniasz PD. Identification of a receptor for an extinct virus. Proc Natl Acad Sci U S A. 2010;107(45):19496-19501.

159. Stanley W. Isolation of a crystaline protein possessing the properties of tobacco-mosaic virus. *Science*. 1935;81:644-645.

160. Stehelin D, Varmus HE, Bishop JM, Vogt PK. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. Nature. 1976;260(5547):170-173.

161. Straub OC. Maedi-Visna virus infection in sheep. History and present knowledge. Comp Immunol Microbiol Infect Dis. 2004;27(1):1-5.

162. Streisinger G, Edgar RS, Denhardt GH. Chromosome structure in phage T4. I. circularity of the linkage map. Proc Natl Acad Sci U S A. 1964;51:775-779.

163. Suttle CA. Viruses in the sea. Nature. 2005;437(7057):356-361.

164. Suttle CA. Marine viruses--major players in the global ecosystem. Nat Rev Microbiol. 2007;5(10):801-812.

165. Szpara ML, Kobiler O, Enquist LW. A common neuronal response to alphaherpesvirus infection. J Neuroimmune Pharmacol. 2010;5(3):418-427.

166. Takahashi WN, Rawlins RE. Method for determining shape of colloidal particles: applications in study of tobacco mosaic virus. *Proc Natl Acad Sci U S A*. 1932;30:155-157.

167. Tang JW, Shetty N, Lam TTY, Ellis Hon KL. Emerging, novel, and known influenza virus infections in humans. Infect Dis Clin North Am. 2010;24(3):603-617.

168. Tang P, Chiu C. Metagenomics for the discovery of novel human viruses. Future Microbiol. 2010;5(2):177-189.

169. Taniguchi T, Palmieri M, Weissmann C. A Qbeta DNA-containing hybrid plasmid giving rise to Qbeta phage formation in the bacterial host [proceedings]. Ann Microbiol (Paris). 1978;129B(4):535-536.

170. Taubenberger JK, Kash JC. Influenza virus evolution, host adaptation, and pandemic formation. Cell Host Microbe. 2010;7(6):440-451.

171. Temin HM, Mizutani S. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature. 1970;226(5252):1211-1213.

172. Thomas CA Jr. The arrangement of information in DNA molecules. J Gen Physiol. 1966;49(6):143-169.

173. Tulsiani SM, Graham GC, Moore PR, et al. Emerging tropical diseases in Australia. Part 5. Hendra virus. Ann Trop Med Parasitol. 2011;105(1):1-11.

174. Twort FW. An investigation on the nature of the ultramicroscopic viruses. *Lancet*. 1915;189:1241-1243.

175. Twort FW. The bacteriophage: the breaking down of bacteria by associated filter-passing lysins. *Br Med*. 1922;2:293.

176. Twort FW. The discovery of the bacteriophage. *Sci News*. 1949;14:33.

177. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. Blood. 1977;50(3):481-492.

178. Venter JC, Smith HO, Adams MD. The sequence of the human genome. Clin Chem. 2015;61(9):1207-1208.

179. Vinson CG, Petre AW. Mosaic disease of tobacco. *Bot Gaz*. 1929;87:14-38.

180. Wang D, Urisman A, Liu Y-T, et al. Viral discovery and sequence recovery using DNA microarrays. PLoS Biol. 2003;1(2):E2.

181. Watson JD, Crick FH. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature*. 1953;171:737-738.

182. Weil PA, Luse DS, Segall J, Roeder RG. Selective and accurate initiation of transcription at the Ad2 major late promotor in a soluble system dependent on purified RNA polymerase II and DNA. Cell. 1979;18(2):469-484.

183. Weil R, Vinograd J. The cyclic helix and cyclic coil forms of polyoma viral DNA. *Proc Natl Acad Sci U S A*. 1963;50:730-738.

184. Weiss R. RNA Tumor Viruses. Cold Spring Harbor Laboratory Press; 1982.

185. Werness BA, Levine AJ, Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science. 1990;248(4951):76-79.

186. Whyte P, Buchkovich KJ, Horowitz JM, et al. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature. 1988;334(6178):124-129.

187. Willner D, Furlan M, Haynes M, et al. Metagenomic analysis of respiratory tract DNA viral communities in cystic fibrosis and non-cystic fibrosis individuals. PLoS One. 2009;4(10):e7370.

188. Willner D, Thurber RV, Rohwer F. Metagenomic signatures of 86 microbial and viral metagenomes. Environ Microbiol. 2009;11(7):1752-1766.

189. Wolbach SB. The filterable viruses, a summary. J Med Res. 1912;27(1):1-25.

190. Woodruff AM, Goodpasture EW. The susceptibility of the chorio-allantoic membrane of chick embryos to infection with the fowl-pox virus. Am J Pathol. 1931;7(3):209-222.5.

191. Worcel A, Burgi E. On the structure of the folded chromosome of Escherichia coli. J Mol Biol. 1972;71(2):127-147.

192. Wyatt GR, Cohen SS. The bases of the nucleic acids of some bacterial and animal viruses: the occurrence of 5-hydroxymethylcytosine. *Biochem J*. 1953;55:774-782.

193. Zhao R, Gish K, Murphy M, et al. Analysis of p53-regulated gene expression patterns using oligonucleotide arrays. Genes Dev. 2000;14(8):981-993.

194. Zinder ND, Lederberg J. Genetic exchange in Salmonella. J Bacteriol. 1952;64(5):679-699.

195. Zinkernagel RM, Doherty PC. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature. 1974;248(5450):701-702.

CHAPTER 2

Principles of Virology

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Virus taxonomy

History and rationale

Current taxonomy

Nomenclature

Informal groupings and alternate classification schemes

Virus cultivation and assay

Initial detection and isolation

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Quantitative assay of viruses

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Phenotypic mixing and pseudotypes

Viruses are unique in nature. They are the smallest of all self-replicating organisms, historically characterized by their ability to pass through filters that retain even the smallest bacteria. In their most basic form, viruses consist solely of a small segment of nucleic acid encased in a simple protein shell. Viruses have no metabolism of their own but rather are obliged to invade cells and parasitize subcellular machinery. Many have argued that viruses are not even living.[166]

The apparent simplicity of viruses is deceptive. The truth is that as a group, viruses infect virtually every organism in nature, they display a dizzying diversity of structures and lifestyles, and they embody a profound complexity of function.

The study of viruses—virology—must accommodate both the uniqueness and the complexity of these organisms. The singular nature of viruses has spawned novel methods of classification and experimentation entirely peculiar to the discipline of virology. The complexity of viruses is constantly challenging scientists to adjust their thinking and their research to describe and understand some new twist in the central dogma revealed in a *simple* virus infection.

This chapter explores several concepts fundamental to virology as a whole, including virus taxonomy, virus cultivation and assay, and virus genetics. The chapter is not intended as a comprehensive or encyclopedic treatment of these topics, but rather as a relatively concise overview with sufficient documentation for more in-depth study. In addition to primary resources and practical experience, the presentation draws heavily on previous editions of *Fields Virology*[46-48,87-89] for the taxonomy and genetics material, plus several excellent texts for material on virus cultivation and assay.[28,45,52,76,93,100,106] It is hoped that this chapter will be of value to anyone learning virology at any stage: a novice trying to understand basic principles for the first time, an intermediate student of virology trying to understand the technical subtleties of virological protocols in the literature, or a bewildered scientist in the laboratory wondering why the host-range virus mutant received from a colleague does not seem to manifest the described host range.

Virus Taxonomy

A coherent and workable system of classification—a taxonomy—is a critical component of the discipline of virology. However, the unique nature of viruses has defied the strict application of many of the traditional tools of taxonomy used in other areas of biology. Thus, scientists who concern themselves with global taxonomy of organisms have traditionally either ignored viruses completely as nonliving entities or left them

scattered throughout the major kingdoms, reasoning that viruses have more in common with their individual hosts than they do with each other.[107,112] By contrast, for practical reasons at least, virologists agree that viruses should be considered together as a separate group of organisms regardless of host, be it plant, animal, fungus, protist, or bacterium. This philosophy is justified by the observation that, in several cases, viruses now classified in the same family—for example, family *Reoviridae*—infect hosts from different kingdoms. Interestingly, the discipline of virus taxonomy brings out thought-provoking and philosophical discussions about the nature of viruses, probably because the decisions that must be made to distinguish one virus from another require deep thought about the nature of viruses and virus evolution. In the end, all of nature is a continuum, and the business of taxonomy has the unfortunate obligation of drawing boundaries within this continuum, an artificial task but necessary nevertheless. The execution of this obligation results today in a free-standing virus taxonomy, overseen by the International Committee on Taxonomy of Viruses (ICTV), with rules and tools unique to the discipline of virology. The process of virus taxonomy that has evolved uses some of the hierarchical nomenclature of traditional taxonomy. However, to cope with both the uniqueness and diversity of viruses as a group, the classification process has been deliberately nonsystematic and thus is "based upon the opinionated usage of data."[114]

Most importantly, the virus taxonomy that has been developed and refined works well. For the trained virologist, the mention of a virus family or genus name, such as "family *Herpesviridae*" or "genus *Rotavirus*" immediately conjures a set of characteristics that form the basis for further description. Virus taxonomy serves an important practical purpose as well, in that the identification of a limited number of biological characteristics, such as virion morphology, genome structure, or antigenic properties, quickly provides a focus for identification of an unknown agent for the clinician or epidemiologist and can significantly impact treatment or prevention of a virus disease. Virus taxonomy is an evolving field, and what follows is a summary of the state of the art, including important historical landmarks that influenced the present system of virus taxonomy and major changes in viral classification over the past 5 years.

History and Rationale

Virology as a discipline is scarcely 100 years old, and thus, the discipline of virus taxonomy is relatively young. In the early 1900s, viruses were initially classified as distinct from other organisms simply by their ability to pass through unglazed porcelain filters known to retain the smallest of bacteria. As increasing numbers of filterable agents became recognized, they were distinguished from each other by the only measurable properties available, namely the disease caused in an infected organism. Therefore, animal viruses that caused liver pathology were grouped together as hepatitis viruses, and viruses that caused mottling in plants were grouped together as mosaic viruses. In the 1930s, an explosion of technology spawned a description of the physical properties of many viruses, providing numerous new characteristics for distinguishing viruses one from another. The technologies included procedures for purification of viruses, biochemical characterization of purified virions, serology, and perhaps most importantly, electron microscopy, which permitted detailed descriptions of virion morphology. In the 1950s, these characterizations led to the distinction of three major animal virus groups, the myxoviruses, the herpesviruses, and the poxviruses.

By the 1960s, because of the profusion of data describing numerous different viruses, it became clear that an organized effort was required to classify and name viruses, and thus the ICTV (originally the International Committee on Nomenclature of Viruses [ICNV]) was established in 1966. The ICTV functions today as a large, international group of virologists organized into appropriate study groups, who develop rules for the classification and naming of viruses and coordinate implementation of these rules. The ICTV is a committee of the Virology Division of the International Union of Microbiological Societies. The objectives of the ICTV are to develop an internationally agreed taxonomy and nomenclature for viruses, to maintain an index of virus names, and to communicate the proceedings of the committee to the international community of virologists. The ICTV publishes an update of the taxonomy at ~3-year intervals. Since 2017, the ICTV Taxonomy Report is freely available online at http://www.ictvonline.org/Report.

Early in its history, the ICTV wrestled with the fundamental problem of developing a taxonomic system for classification and naming of viruses that would accommodate the unique properties of viruses as a group and that could anticipate advancements in the identification and characterization of viruses. Perhaps the most critical issue was whether the classification of viruses should consider virus properties in a monothetical or polythetical fashion. A *monothetic* system of classification is defined as a system based on a single characteristic or a series of single characteristics. *Polythetic* is defined as sharing several common characteristics without any one of these characteristics being essential for membership in the group. Thus, a monothetical classification, modeled after the Linnaean system used for classification of plants and animals, would rank individual virus properties, such as genome structure or virion symmetry, as being more or less important relative to each other and use these individual characteristics to sort viruses.[103] Although the hierarchical ordering of viruses is desirable, a strictly monothetical approach was problematic because both the identification of individual properties and the assignment of a hierarchy to individual properties seemed too arbitrary. A polythetic approach to classification would group viruses by comparing simultaneously numerous properties of individual viruses without assigning a universal priority to any one property. Thus, using the polythetic approach, a given virus grouping is defined by a collection of properties. One argument against the polythetic approach is that a truly systematic and comprehensive comparison of dozens of individual properties would be difficult if not impossible. However, this problem could be avoided by the adoption of a nonsystematic approach, using study groups of virologists within the ICTV to consider together numerous characteristics of a virus and make as rational an assignment to a group as possible. Therefore, the system that is currently being used is a nonsystematic, polythetical, hierarchical system. This system differs from any other taxonomic system in use for bacteria or other organisms; however, it is effective and useful.[113] As new techniques for identification and characterization of viruses are developed, the methods used for taxonomy continue to evolve, as described below.

Taxonomy Prior to 2018

Due to their high mutation rates, limited number of genes, and relative lack of old/"fossil" records, it was originally thought that deep evolutionary relationships among viruses could not be determined. As a consequence, prior to 1990, the highest taxonomic rank for viruses was family, followed by subfamily, genera, and species. This original viral classification strategy was completely separate from the tree of cellular life and only included the lower levels of the classical hierarchy, omitting designations for kingdom, phylum, and class. In practice, this original taxonomy was filled initially from the middle of the hierarchy by assigning viruses to genera and then elaborating the taxonomy upward by grouping genera into families and, to a limited extent, families into orders. By 1970, the ICTV had established two virus families each containing 2 genera, 24 floating genera, and 16 plant *groups*.[174] A rigorous species definition was not approved by the ICTV until 1991 but was then applied to the entire taxonomy and became the primary level of classification for viruses.[165] By 2011, the ICTV recognized 6 orders, 87 families, 19 subfamilies, 348 genera, and 2290 species.

For most experimental virologists, the viral family and species designations are among the most useful and familiar because they describe a

given morphology and replication strategy (family) and they are part of the vernacular (species). For example, *West Nile virus* is a species within the *Flaviviridae* family, *Vaccinia virus* is a species within the *Poxviridae* family, and *Measles morbillivirus* is a species with the *Paramyxoviridae* family. Until changes enacted in 2018, viral family was the highest consistently used taxonomic grouping, and therefore carries a convenient generalized description of a given virus group, and as a result has become a benchmark of the taxonomic system. Most families have distinct virion morphology, genome structure, and/or replication strategy (see Table 2.1). A virus species is defined as "a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche."[164] The formal definition of a polythetic class is "a class whose members always have several properties in common, although no single common attribute is present in all of its members."[165] Thus, no single property can be used to define a given species, and application of this formal definition of a polythetic class to species accounts nicely for the inherent variability found among members of a species. The qualification of a replicating lineage implies that members of a species experience evolution over time with consequent variation but that members share a common ancestor. The qualification of occupation of an ecological niche acknowledges that the biology of a virus, including such properties as host range, pathogenesis, transmission, and habitat, are fundamental components of the characterization of a virus. Taxonomic levels lower than species, such as clades, strains, and variants, are not officially considered by the ICTV but are left to specialty groups.

TABLE 2.1 Characteristics of a small subset of vertebrate virus families

Family	Virion Morphology	Envelope	Genome[a]
dsDNA viruses			
Adenoviridae	Icosahedral	No	1 ds linear
Herpesviridae	Spherical, tegument	Yes	1 ds linear
Papillomaviridae	Icosahedral	No	1 ds circular
Polyomaviridae	Icosahedral	No	1 ds circular
Poxviridae	Ovoid	Yes	1 ds linear
ssDNA viruses			
Anelloviridae	Icosahedral	No	1 (–) circular
Circoviridae	Icosahedral	No	1 (– or +/–) circular
Parvoviridae	Icosahedral	No	1 (+, –, or +/–) circular
dsDNA reverse-transcribing viruses			
Hepadnaviridae	Spherical	Yes	1 ds circular
ssRNA reverse-transcribing viruses			
Retroviridae	Spherical	Yes	1 (+) linear
dsRNA viruses			
Reoviridae	Icosahedral	No	10-12 ds linear
Negative-sense ssRNA viruses			
Filoviridae	Filamentous	Yes	1 (–) linear
Orthomyxoviridae	Pleomorphic, spherical	Yes	6-8 (–) linear
Paramyxoviridae	Pleomorphic spherical	Yes	1 (–) linear
Rhabdoviridae	Bullet shaped	Yes	1 (–) linear
Positive-sense ssRNA viruses			
Arteriviridae	Spherical	Yes	1 (+) linear
Astroviridae	Icosahedral	No	1 (+) linear
Caliciviridae	Icosahedral	No	1 (+) linear
Coronaviridae	Spherical	Yes	1 (+) linear
Flaviviridae	Spherical	Yes	1 (+) linear
Picornaviridae	Icosahedral	No	1 (+) linear
Togaviridae	Spherical	Yes	1 (+) linear
Ambisense ssRNA viruses			
Arenaviridae	Spherical	Yes	2 (+/–) linear
Bunyaviridae	Spherical	Yes	3 (– or +/–) linear

[a]Number of segments, polarity (ds, double stranded; +, mRNA-like; –, cRNA-like; +/–, ambisense), conformation.

Taxonomic levels higher than species are formally defined by the ICTV only in a relative sense, namely a genus is a group of species sharing certain common characters, a subfamily is a group of genera sharing certain common characters, a family is a group of genera or subfamilies sharing certain common characters, and so forth. As the virus taxonomy has evolved, these higher taxa have acquired some monothetic character. They remain polythetic in that they may be characterized by more than one virus property; however, they violate the formal definition of a polythetic class in that one or more defining properties may be required of all candidate viruses for membership in the taxon. Not all taxonomic levels need be used for a given grouping of viruses; thus, whereas most species are grouped into genera and genera into families, not all families contain subfamilies, and only a few families have been grouped into orders.

Historically, a *type species* was identified for each genus. The type species is not necessarily the best characterized or most representative species in a genus; rather, it is usually the virus that initially necessitated the creation of the genus and therefore best defines or identifies the genus. However, in 2021, the ICTV decided to abolish the notion of a type species because it was mainly historical and no longer served a clear purpose.[169]

Current Taxonomy

A major shift in virus taxonomy has occurred over the past few years. The strategic change is in part due to the explosion of newly discovered viruses through metagenomic approaches but also due to recognition that taxonomy can be extended to include shared origins and evolutionary relationships among even distantly related viruses. Now, rather than a five-rank structure at the lower rungs of the evolutionary hierarchy (order, family, subfamily, genera, species), current taxonomy uses a 15-rank structure that more closely parallels cell-based organisms (realm, subrealm, kingdom, subkingdom, phylum, subphylum, class, subclass, order, suborder, family, subfamily, genus, subgenus, species). An important distinction was made at the top of the viral hierarchy to distinguish it from a similar rung on the evolutionary tree of cell-based organisms. The ICTV designated "realms" for viruses that are analogous to "domains" for bacteria, archaea, and eukaryota. Unlike cell-based organisms, viruses do not share one common ancestor and therefore phylogenetic trees cannot be made. Thus, the ICTV invented realms to convey similarity of genes and proteins, even when members arose from different ancestors. As of 2020, there are six realms: *Riboviria*, *Duplodnaviria*, *Monodnaviria*, *Varidnaviria*, *Adnaviria*, and *Ribozyviria*. The realm names are usefully descriptive. *Riboviria* are RNA viruses that use an RNA-directed polymerase to replicate, *Duplodnaviria* are dsDNA viruses with a common HK97-fold capsid protein, *Monodnaviria* are ssDNA viruses with a common endonuclease, *Varidnaviria* are DNA viruses with a common jelly roll capsid protein, *Adnaviria* are filamentous viruses with A-form DNA that infect archaea, and *Ribozyviria* are unusual small RNA viruses. In addition to these 6 realms, in 2020, there were 10 kingdoms, 17 phyla, 2 subphyla, 39 classes, 59 orders, 8 suborders, 189 families, 136 subfamilies, 2224 genera, 70 subgenera, and 9110 species.

While the higher level ranks—realm, kingdom, phyla, and class—were created in 2018 and are therefore new, the lower level ranks—order, family, genera, and species—have remained fairly constant aside from addition of new members from metagenomic studies. Thus, genus and species designations for existing viral systems have not changed with the new taxonomy. As a consequence, many virologists working on established viral systems may be unaware of the changes in taxonomy. *West Nile virus* is still *West Nile virus*, it just has a new realm (*Riboviria*), kingdom (*Orthornavirae*), phylum (*Kitrinoviricota*), and class (*Flasuviricetes*).

To facilitate the management and distribution of virological data, the ICTV shares taxonomic and other information on their web site: https://talk.ictvonline.org/. Constructed from virus descriptions in the published reports of the ICTV, the database comprises searchable descriptions, updated taxonomy, and historical records. The complete virus taxonomy is far too extensive to relate here; however, Figure 2.1 shows the general scheme and one specific example, *Zaire ebolavirus*.

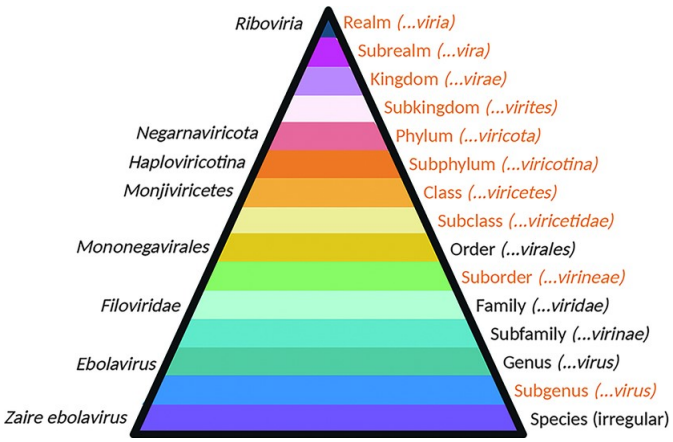

FIGURE 2.1 **Current taxonomy.** Viruses are grouped into 15 taxonomic ranks as shown on the *right side* of the diagram. Ranks indicated in *black text* were part of the prior scheme and ranks indicated in *orange text* were added in 2018. The width of the triangle indicates relative numbers within each rank, with 6 realms and over 9000 species in 2021. A specific example for one virus, *Zaire ebolavirus*, is shown on the *left*. (Created with BioRender.com.)

Metagenomics

Viral metagenomics is the study of genetic material collected directly from the environment, without the need to isolate or culture viruses, and it has dramatically expanded the known virosphere. Thousands of new viral genomes from diverse environmental sites can be sequenced quickly, and a method for taxonomic classification is needed. The ICTV accepts proposals to create taxa on the basis of genomic sequences alone—without the need for host information or other biological information—as long as there is evidence for accurate sequence assembly and the coding sequence is complete.[1,148] Additionally, it is possible to create species solely on the basis of sequencing data using the same hierarchy and nomenclature rules as traditionally described species. This has been particularly useful for an explosion of viral sequences from marine and intestinal environments.

Nomenclature

The ICTV has adopted a formal nomenclature for viruses, specifying suffixes for the various taxa, and rules for written descriptions of viruses. Names for each category must be single words ending with an appropriate suffix: realm (-viria), subrealm (-vira), kingdom (-virae), subkingdom (-virites), phylum (-viricota), subphylum (-viricotina), class (-viricetes), subclass (-viricetidae), order (-virales), suborder (-virinae), family (-viridae), subfamily (-virinae), genus (-virus), and subgenus (-virus). Species names may contain more than one word and have no specific

ending. In written usage, the formal virus taxonomic names are capitalized and written in italics, and preceded by the name of the taxon, which is neither capitalized nor italicized. For species names that contain more than one word, the first word plus any proper nouns are capitalized. As an example, the full formal written description of human immunodeficiency virus 1 is as follows: realm *Riboviria*, kingdom *Pararnavirae*, phylum *Artverviricota*, class *Revtraviricetes*, order *Ortervirales*, family *Retroviridae*, subfamily *Orthoretrovirinae*, genus *Lentivirus*, species *Human immunodeficiency virus 1*. The ICTV acknowledges that vernacular (informal) taxonomic names are widely used; however, they should not be italicized or capitalized except for proper names. For example, the vernacular name "herpesvirus" refers to a member of the family *Herpesviridae*.

While viral *taxa* are officially named by the ICTV, virus *names* are chosen by the virologists that discover and study them. In practice, the chosen names are valid only if they are accepted and used by the relevant virology community. Technically, there are no rules to govern virus names, but historically, viruses have been named after their properties, locations, hosts, diseases, or their discoverers. More recently, naming viruses after locations has been discouraged to avoid stigma associated with an outbreak. Thus, while existing virus names may refer to locations (Norwalk virus, Ebola virus, Japanese encephalitis virus, etc.), more newly discovered viruses are not named after locations (eg, SARS-CoV-2).

Informal Groupings and Alternate Classification Schemes

For convenience in presenting or tabulating the virus taxonomy, informal categorical groupings of taxa are often used. The criteria typically include nature of the viral genome (DNA or RNA), strandedness of the viral genome (single-stranded or double-stranded), polarity of the genome (positive sense, negative sense, or ambisense), and reverse transcription. Separate categories accommodate subviral agents (including viroids, satellites, and prions) and unassigned viruses. The Baltimore classification system, named after its creator David Baltimore, is a widely used scheme based on the nature of the genome packaged in virions and the pathway of nucleic acid synthesis that each group takes to accomplish messenger RNA (mRNA) synthesis.[3] This classification divides viruses into seven categories as depicted in Figure 2.2. Most usages of this system group ambisense virus families (family *Arenaviridae* and family *Bunyaviridae*) along with negative-sense single-stranded RNA (ssRNA) viruses. The families of vertebrate viruses listed in Table 2.1 have been grouped according to the Baltimore classification, with ambisense viruses split into an eighth genome category.

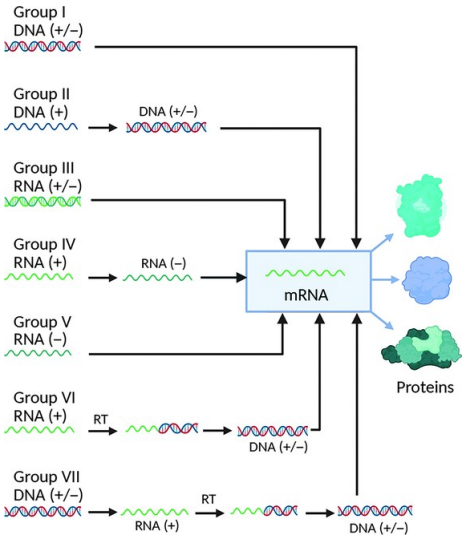

FIGURE 2.2 **The Baltimore classification system, a virus classification scheme based on the form of nucleic acid present in virion particles and the pathway for expression of the genetic material as messenger RNA.**[3] Viruses containing ambisense single-stranded RNA genomes are grouped under negative-sense single-stranded RNA viruses. +, sense strand; –, antisense strand; +/–, double-stranded; RT, reverse transcription. (Created with BioRender.com.)

Virus Cultivation and Assay

A genuine understanding of virology requires a clear understanding of virological methods. Some of these methods use techniques shared by other branches of biological science, whereas others are generally quite unique to the study of viruses. What follows is a summary of common techniques used in virology, presented as fundamental background for understanding the discipline.

Initial Detection and Isolation

The presence of a virus is typically assessed by examining effects on a host organism or, in the case of many animal viruses, by effects on cultured cells. Effects on animal hosts include a broad range of symptoms such as virus-induced lesions, behavioral changes, immune dysfunction tissue damage, organ failure, and death. Effects on cultured cells include a variety of morphological changes in infected cells, termed *cytopathic effects*, and described in detail later in this chapter. Both adenovirus[138] and the polyomavirus SV40[158] were discovered as cell culture contaminants before they were detected in their natural hosts.

Viruses can be isolated from an infected host by harvesting excreted or secreted material, blood, or tissue and testing for induction of the original symptoms in the identical host, or induction of some abnormal pathology in a substitute host or in cell culture. Historically, dogs, cats, rabbits, rats, guinea pigs, hamsters, mice, and chickens have all been found to be useful in laboratory investigations,[93] although most animal methods have now been replaced by cell culture methods.[106] There is also growing interest in identifying and isolating viruses from their natural hosts or from environmental samples (eg, sea water). Modern metagenomic sequencing techniques can be used to assess the repertoire of viral sequences in a given sample, also referred to as the "virome."[49]

Once the presence of a virus has been established, it is often desirable to prepare a genetically pure clone. This can be achieved by either limiting serial dilution or by plaque purification of a verified viral stock. Alternatively, many viral genomes, particularly RNA viruses, can be synthesized *de novo* using recombinant DNA technologies, as shown initially with poliovirus.[12]

Viruses that are cultivated in anything other than the natural host may adapt to the new host through acquisition of genetic alterations that provide a replication advantage in the new host. Such adaptive changes may be accompanied by a loss of fitness in the original host, most notably by a loss of virulence or pathogenicity. Whereas this adaptation and attenuation may present problems to the basic scientist interested in understanding the replication of the virus in its natural state, it also forms the basis of construction of attenuated viral vaccines.

Hosts for Virus Cultivation

Laboratory Animals and Embryonated Chicken Eggs

Prior to the advent of cell culture, animal viruses were propagated in whole animals or embryonated chicken eggs. Whole animals could include the natural host or laboratory animals such as rabbits, mice, rats, and hamsters. In the case of laboratory animals, newborn or suckling rodents often provide the best hosts. Today, laboratory animals are seldom used for routine cultivation of virus; however, they still play an essential role in studies of viral pathogenesis.

The use of embryonated chicken eggs was introduced to virology by Goodpasture et al.[56] in 1932 and developed subsequently by Beveridge and Burnet.[6] The developing chick embryo, 10-14 days after fertilization, provides a variety of differentiated tissues, including the amnion, allantois, chorion, and yolk sac, which serve as substrates for growth of a wide variety of viruses, including orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, herpesviruses, and poxviruses.[93] These virus may replicate in several tissues of the developing egg, or replication may be confined to a single tissue. Some of them cause discrete and characteristic foci when introduced onto the chorioallantoic membrane of embryonated eggs, thus providing a method for identification of virus types or for quantifying virus stocks or assessing virus pathogenicity. Although embryonated eggs have been almost wholly replaced by cell culture techniques, they are still the most convenient method for growing high titer stocks of some viruses and thus continue to be used both in research laboratories and for vaccine production.

Cell Culture

The growth and maintenance of animal cells *in vitro*, described generally as tissue culture, can be divided into several different techniques: organ culture, primary explant culture, organoid culture, and cell culture. In *organ culture*, the original three-dimensional architecture of a tissue is preserved under culture conditions that provide a gas-liquid interface. In *primary explant culture*, minced pieces of tissue placed in liquid medium in a culture vessel provide a source for outgrowth of individual cells. *Organoid cultures* are a more recent addition to the repertoire of tissue culture and involve the formation of three-dimensional organlike structures from one or more stem cell types that differentiate to mimic the corresponding organ *in vivo*. In *cell culture*, tissue is disaggregated into individual cells prior to culturing. Cell culture and organoid culture will be discussed in more detail here, as they are the most commonly used tissue culture techniques in virology.

Cultured cells currently provide the most widely used and most powerful hosts for cultivation and assay of viruses. Cell cultures are of three basic types—primary cell cultures, cell strains, and cell lines—that may be derived from many animal species and that differ substantially in their characteristics. Viruses often behave differently on different types of cultured cells; in addition, each of the culture types possesses technical advantages and disadvantages. For these reasons, an appreciation of the use of cultured cells in animal virology requires an understanding of several fundamentals of cell culture itself. A detailed description of the theory and practice of cell and tissue culture is provided by Freshney,[52] and several additional texts provide excellent summaries of cell culture as it specifically applies to virology.[28,76]

Primary cell culture

A primary cell culture is defined as a culture of cells obtained from the original tissue that have been cultivated *in vitro* for the first time and that have not been subcultured. Primary cell cultures can be established from whole animal embryos or from selected tissues from embryos, newborn animals, or adult animals of almost any species. The most commonly used cell cultures in virology derive from primates, including humans and monkeys; rodents, including hamsters, rats, and mice; and birds, most notably chickens. Cells to be cultured are obtained by mincing tissue and dispersing individual cells by treatment with proteases and/or collagenase to disrupt cell-cell interactions and interactions of cells with the extracellular matrix. With the exception of cells from the hemopoietic system, normal vertebrate cells will grow and divide only when attached to a solid surface. Dispersed cells are therefore placed in a plastic flask or dish, the surface of which has been treated to promote cell attachment. The cells are incubated in a buffered nutrient medium in the presence of blood serum, which contains a complex mixture of hormones and factors required for the growth of normal cells. The blood serum may come from a variety of sources, although bovine serum is most commonly used. Under these conditions, cells will attach to the surface of the dish, and they will divide and migrate until the surface of the dish is covered with a single layer of cells, a monolayer, whereupon they will remain viable but cease to divide. If the cell monolayer is "wounded" by scraping cells from an isolated area, cells on the border of the wound will resume division and migration until the monolayer is reformed, whereupon cell division again ceases. These and other observations lead to the conclusion that the arrest of division observed when cells reach confluency results from cell-cell contact and therefore is called *contact inhibition*. Primary cultures may contain a mixture of cell types and retain the closest resemblance to the tissue of origin.

Subcultivation

Cells from a primary culture may be subcultured to obtain larger numbers of cells. Cells are removed from the culture dish and disaggregated by treating the primary cell monolayer with a chelating agent, usually EDTA, or a protease, usually trypsin, or both, giving rise to a single cell suspension. This suspension is then diluted to a fraction of the original monolayer cell density and placed in a culture dish with fresh growth medium, whereupon the cells attach to the surface of the dish and resume cell division until once again a monolayer is formed and cell division ceases. Cultures established in this fashion from primary cell cultures may be called *secondary cultures*. Subsequently, cells may be repeatedly subcultured in the same fashion. Each subculturing event is called a *passage*, and each passage may comprise several cell generations, depending on the dilution used during the passage. Most vertebrate cells divide at the rate of approximately one doubling every 24 hours at 37 °C. Thus, a passage performed with an eightfold dilution will require three cell doublings over 3 days before the cells regain confluency.

Cell strains

Normal vertebrate cells cannot be passaged indefinitely in culture. Instead, after a limited number of cell generations, usually 20-100 depending on the age and species of the original animal, cultured normal cells cease to divide, then degenerate and die, a phenomenon called *crisis* or *senescence*.[65] Starting with the establishment of a secondary culture and until cells either senesce or become transformed as described later, the culture is termed a *cell strain* to distinguish it from a primary culture on the one hand, or a transformed, immortal cell line on the other hand. During culture, cells in a strain retain their original karyotype and are thus called *euploid*; however, culturing induces profound changes in the composition and characteristics of the cell strain, which are manifested early during the passage history and may continue during passage. Whereas primary cell cultures may contain a mixture of cell types that survive the original plating of cells, only a few cell types survive subculturing; thus, by the second or third passage, typically only one cell type remains in the cell strain. Cell strains are usually composed of one of two basic cell types—fibroblast-like or epithelial-like—characterized based on their morphology and growth characteristics (Fig. 2.3). Fibroblasts have an elongated, spindle shape, whereas epithelial cells have a polygonal shape. Although after only a few passages only one cell type may remain in a cell strain, continued passage may select for faster-growing variants, such that the characteristics of a cell strain may change with increasing passage number. Despite the fact that normal cell strains experience senescence in culture, they may be maintained for many years by expanding the culture to a large number of cells early during the passage history and storing numerous small samples of low passage cells by freezing. Therefore, as a given strain approaches high passage number and senescence, low passage cells of the same strain may be thawed and cultured.

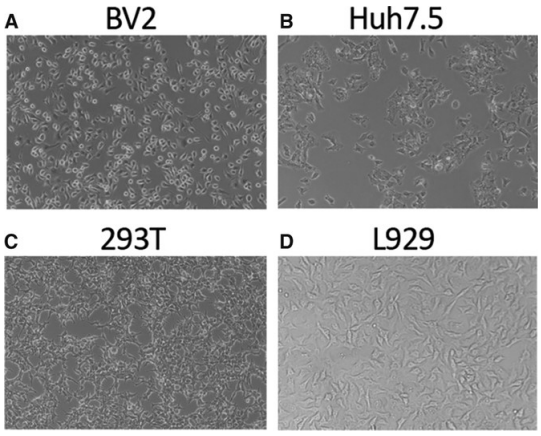

FIGURE 2.3 **Cultured cell types**. Phase contrast photomicrographs of subconfluent monolayers are shown. **A**. BV2 mouse microglia macrophage-like cells. **B**. Huh7.5 human hepatoma cells. **C**. 293T human embryonic kidney cells. **D**. L929 mouse fibroblast cells. (BV2, Huh7.5, and 293T images courtesy of Valerie Rodriguez Irizarry; L929 images courtesy of Dr. Andrea Erickson.)

Cell lines

At any time during the culture of a cell strain, cells in the culture may become *transformed* such that they are no longer subject to crisis and senescence but can be passaged indefinitely. Transformation is a complex phenomenon, discussed in more detail later and in Chapter 11; however, in the context of cell culture, the most important characteristic of transformation is that the transformed cells become immortalized. Immortal cell cultures are called *cell lines*, or sometimes *continuous cell lines*, to distinguish them from primary cultures and cell strains. Immortalization can occur spontaneously during passage of a cell strain, or it can be induced by treatment with chemical mutagens, infection with tumorigenic viruses, or transfection with oncogenes. In addition, cells cultured from tumor tissue often establish immortal cell lines in culture. Spontaneous immortalization does not occur in cultured cells from all animal species. It has been observed frequently during culture of rodent cells (eg, in mouse and hamster cell strains), to a lesser extent in monkey kidney cells, and rarely, if at all, during the culture of chicken or human cells. Immortalization is typically accompanied by genetic changes such that cells become aneuploid, and the karyotype of each cell often differs across the culture.

As with the propagation of cell strains, continued culture of a cell line may result in selection of specific variants that outgrow other cells over time. With passage, the character of a cell line may change substantially, and cell lines of the same origin cultured in different laboratories over a period of years may have significantly different characteristics. It is prudent, therefore, to freeze stocks of cell lines having specific desirable properties so that these cells can be recovered if the properties disappear during culture. Likewise, it makes sense to obtain a cell line showing certain desired characteristics directly from the laboratory that described those characteristics, because cells from alternate sources may differ in character.

Transformation

Transformed cells are distinguished from normal cells by numerous properties that can be grouped into three fundamental types of changes: immortalization, aberrant growth control, and malignancy. *Immortalization* refers simply to the ability to be cultured indefinitely, as described previously. *Aberrant growth control* comprises a number of properties, several of which have relevance to experimental virology, including loss of contact inhibition, anchorage independence, and tumorigenicity. Loss of contact inhibition means that cells no longer cease to grow as soon as a monolayer is formed, and cells will now grow on top of one another. Anchorage independence means that the cells no longer need to attach to a solid surface to grow. Anchorage independence is often assayed as the ability to form colonies suspended in a semisolid medium such as agar, and a practical consequence of anchorage independence is the ability to grow in liquid suspension. Tumorigenicity refers to the ability of cells to form a tumor in an experimental animal, and *malignancy* refers to the ability to form an invasive tumor *in vivo*. While malignancy is obviously of vital importance as a phenomenon in its own right, it has limited application in virology except within the specific discipline of tumor virology (Chapter 11). Importantly, the many properties of transformed cells are not necessarily interdependent, and no one property is an absolute prerequisite for another. Thus, transformation is thought to be a multistep genetic phenomenon, and varying degrees of transformation are measurable. Tumorigenicity is often regarded as the most stringent assay for a fully transformed cell and is most closely correlated with

anchorage independence.

The fact that the various characteristics of transformed cells are not interdependent has important consequences for experimental virology, especially in the assay of tumor viruses. Specifically, a transformed cell line that is immortalized but still contact inhibited may be used in a viral transformation assay that measures the further transformation to loss of contact inhibition. When cells in a monolayer are transformed by a tumor virus and lose contact inhibition, they grow on top of a confluent monolayer, forming a *focus*, literally a pile of cells, which is readily distinguishable from the rest of the monolayer. This property forms the basis of a quantitative assay in which foci formed by tumor viruses can be stained and directly counted.[167]

Advantages and disadvantages of different cultured cell types

The various types of cultured cells described previously have specific application to different problems encountered in experimental virology. For most applications, an adherent cell line provides the most useful host cell. Cell lines are relatively easy to maintain because they can be passaged indefinitely, and adherence is a prerequisite for a plaque assay, described later. A distinct technical advantage of adherent cells is that the culture medium can easily be changed for the purposes of infection or metabolic labeling by simply aspirating and replacing fluid from a monolayer, a process that requires repeated centrifugations with suspension cells. By contrast, relative to adherent cell lines, suspension cell lines are easier to sample than adherent cells, and they produce large numbers of cells from a relatively small volume of medium in a single culture vessel, which has significant advantages for some high-volume applications in virology. Unfortunately, not all viruses will grow on a cell line, and often under these circumstances, a primary cell culture will suffice. This may reflect a requirement for a particular cell type found only under conditions of primary cell culture, or it may reflect a requirement for a state of metabolism or differentiation closely resembling the *in vivo* situation, which is more likely to exist in a primary culture than it is in a cell line.

Lastly, some viruses do not grow in standard cell culture at all. One example is the papillomaviruses, which cause warts. Although the viral nature of papillomatosis was demonstrated decades ago, progress studying these viruses was hampered because the viruses did not grow in culture. The inability to grow papillomavirus in culture results from a tight coupling of the regulation of viral gene expression with the differentiation state of the target epithelial cell, which in turn is tightly coupled to the three-dimensional architecture of the epidermis, which is lost in culture. Specialized tissue culture techniques have been developed that result in the faithful reconstruction of an epidermis by seeding primary keratinocytes on a “feeder” layer composed of an appropriate cell line and incubating these cells on a “raft” or grid at a liquid-air interface. On these raft cultures, the entire replication cycle of a papillomavirus can be reproduced *in vitro*, albeit with difficulty.[14] More recently, efficient infection of papillomavirus was achieved by prebinding viral particles to extracellular matrix components that were previously deposited by cells cultured in a plastic dish. After virus binding to the matrix, keratinocytes were overlayed and subsequently infected with higher efficiency than in previous models.[7] Notably, in the absence of these cultures, a large fraction of the genetics and biology of papillomaviruses was still able to be determined primarily through the use of recombinant DNA technology, without ever growing virus in culture.

A second and more contemporary example of a virus that has been difficult to culture in cells is human norovirus, a highly contagious foodborne virus that causes severe outbreaks of gastroenteritis worldwide. Human norovirus replicates in intestinal epithelial cells and certain immune cells, but standard culture of those various cell types does not support efficient norovirus replication. One study took advantage of human intestinal enteroid cultures (discussed below) derived from stem cells that are isolated from intestinal crypts from primary human tissue.[41,141] The enteroids supported replication of multiple strains of human norovirus, though certain strains required the presence of human bile for replication. Another group demonstrated that both human and murine norovirus could infect B cells, but that infection was uniquely dependent on the presence of enteric bacteria.[78] These two examples provide compelling support for the general concept that certain seemingly intractable viruses may simply require additional components to convert otherwise noninfectable cells into adequate cultures for viral entry and replication.

Organoids

Organoids are 3D cell cultures that reflect the architecture, genetics, and physiology of a specific organ or tissue type. Organoids can be derived from a variety of stem cells, including embryonic, pluripotent, or tissue-specific progenitors. Specific growth factors and environmental conditions allow the organoid cultures to take on organlike properties, including the ability to be infected by viruses. Indeed, organoids have been developed for multiple viruses and organs, with much of the work focusing on viruses that infect the intestinal tract, brain, or lungs. With respect to intestinal viruses, there are two primary 3D culture systems: organoids and enteroids. Intestinal organoids are stem cell–derived,[151] whereas enteroids are epithelial cell–only cultures derived from intestinal tissue biopsies.[142] These organoids and enteroids have allowed significant advances in the study of rotaviruses, adenoviruses, and enteric viruses from the *Picornaviridae* and *Caliciviridae* families. These advances include but are not limited to new methods for culturing previously intractable viruses, replication properties of clinical isolates, determination of cellular tropism, role of innate immune pathways during infection, and testing biologics and therapeutics for antiviral activity. Figure 2.4 depicts a human intestinal enteroid infected with coxsackievirus B3.

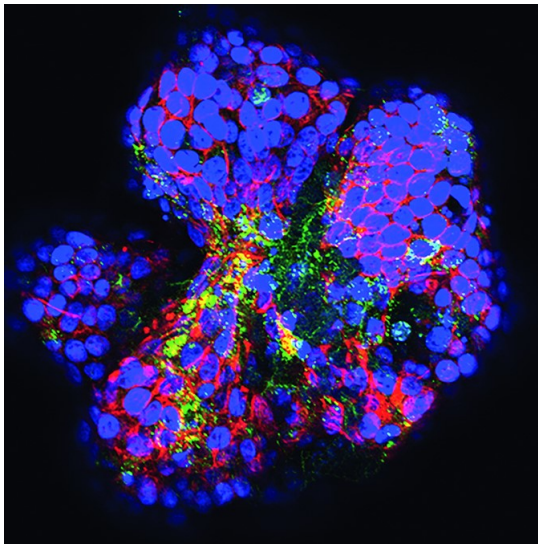

FIGURE 2.4 **Organoid culture.** Human intestinal enteroid organoid system[37] infected with coxsackievirus B3. Nuclei in *blue* (stained with DAPI), dsRNA in *green* (viral replication intermediates, stained with a dsRNA-specific antibody), actin in *red* (phalloidin). (Courtesy of Dr. Carolyn Coyne.)

The development of cerebral organoids predated the Zika virus outbreak of late 2015 by just a few years.[97] Given the link between Zika virus and microcephaly, cerebral organoids derived from embryonic or pluripotent stem cells rapidly became desirable systems for modeling the effects of Zika virus on the developing brain.[54,131] Indeed, these organoids were used to demonstrate both the preferential tropism of Zika virus for neural progenitor cells and the cytotoxic effects of the virus on these cells, thereby recapitulating pathological features observed in clinical microcephaly. Other neurotropic viruses have also been studied in cerebral organoids, including measles virus, La Crosse virus, and herpes simplex virus 1.

In 2017, lung organoids derived from pluripotent stem cells were shown to be viable models for both lung fibrotic disease and respiratory syncytial virus infection.[13] Similarly, lung stem cells were used to develop organoids that, when infected with influenza A virus, recapitulated various features of viral infection in human airway, including cellular tropism, viral replication kinetics, and induction of host innate responses.[73] The COVID-19 pandemic has further catapulted the rapid development and used of lung organoids for a variety of studies on SARS-CoV-2, including basic virological properties and testing of antivirals.[139,160]

Recognition of Viral Growth in Culture

Two classical methods for the recognition of a virus infection in culture are cytopathic effect and hemadsorption. *Cytopathic effect* comprises two different phenomena: (1) morphological changes induced in individual cells or groups of cells by virus infection that are easily recognizable under a light microscope and (2) inclusion bodies, which are more subtle alterations to the intracellular architecture of individual cells. *Hemadsorption* refers to indirect measurement of viral protein synthesis in infected cells, detected by adsorption of erythrocytes to the surface of infected cells. Cytopathic effect is the simplest and most widely used criterion for infection; however, not all viruses cause a cytopathic effect, and in these cases, other methods must suffice.

Morphological changes induced by virus infection comprise a number of cell phenomena, including rounding, shrinkage, increased refractility, fusion, aggregation, loss of adherence, or lysis. Morphological changes caused by a given virus may include several of these phenomena in various combinations, and the character of the cytopathic effect may change reproducibly during the course of infection. Morphological changes caused by a given virus are very reproducible and can be so precisely characteristic of the virus type that significant clues to the identity of a virus can be gleaned from the cytopathic effect alone (Chapter 16). Figure 2.5 depicts different cytopathic effects caused by viruses. Most important to the trained virologist, a simple microscopic examination of a cell culture can reveal whether an infection is present, what fraction of cells are infected, and how advanced the infection is. In addition, because cytopathology results directly from the action of virus gene products, virus mutants can be obtained that are altered in cytopathology, yielding either a conveniently marked virus or a tool to study cytopathology *per se*.

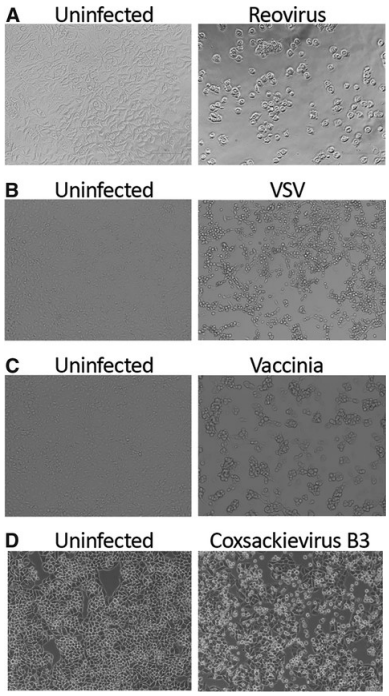

FIGURE 2.5 **Virus-induced cytopathic effects. Phase contrast photomicrographs are shown. A**. Uninfected and reovirus-infected L929 cells at 24 hours postinfection. **B**. Uninfected and vesicular stomatitis virus–infected BSC40 cells at 24 hours postinfection. **C**. Uninfected and vaccinia virus–infected BSC40 cells at 24 hours postinfection. **D**. Uninfected and coxsackievirus B3–infected HeLa cells at 8 hours postinfection. (Reovirus images courtesy of Dr. Andrea Erickson, VSV and vaccinia virus images courtesy of Dr. Don Gammon, and coxsackievirus B3 images courtesy of Robert Maples.)

The term *inclusion bodies* refers generally to the observation of intracellular structures specific to an infected cell and discernible by light microscopy. The effects are highly specific for a particular virus type so that, as with morphological alterations, the presence of a specific type of inclusion body can be diagnostic of a specific virus infection. Electron microscopy, combined with a more detailed understanding of the biology of many viruses, reveals that inclusion bodies usually represent focal points of virus replication and assembly, which differ in appearance depending on the virus. For example, Negri bodies formed during a rabies virus infection represent collections of virus nucleocapsids.[110]

Hemadsorption refers to the ability of red blood cells to attach specifically to virus-infected cells.[146] Many viruses synthesize cell attachment proteins, which carry out their function wholly or in part by binding substituents such as sialic acid that are abundant on a wide variety of cell types, including erythrocytes. Often, these viral proteins are expressed on the surface of the infected cell—for example, in preparation for maturation of an enveloped virus through a budding process. Thus, a cluster of infected cells may be easily detectable to the naked eye as areas that stain red after exposure to an appropriate preparation of red blood cells. Hemadsorption can be a particularly useful assay for detecting infections by viruses that cause little or no cytopathic effect.

Virus Cultivation

The method chosen for growing virus depends on a variety of factors, including (1) the goals of the experiment, namely whether large amounts of one virus variant or small amounts of several variants are to be grown; (2) limitations in the *in vitro* host range of the virus, namely whether it will grow on embryonated eggs, primary cell cultures, continuous adherent cell lines, or suspension cell lines; and (3) the relative technical ease of alternative possible procedures. Furthermore, the precise method for harvesting a virus culture will depend on the biology of the virus—for example, whether it buds from the infected cell, lyses the infected cell, or leaves the cell intact and stays tightly cell associated. As a simple example, consider cultivation of a budding, cytopathic virus on an adherent cell line. Confluent monolayers of an appropriate cell line are exposed to virus diluted to infect a fraction of the cells, and progress of the infection is monitored by observing the development of the cytopathic effect until the infection is judged completely based on experience with the relationship between cytopathic effect and maximum virus yield. A crude preparation of virus can be harvested simply by collecting the culture fluid and freezing down aliquots that can be stored for years, if not indefinitely. In cases where large quantities of virus are needed, for example, for biophysical characterization or structural studies by x-ray crystallography or cryo-EM, virus-containing supernatants can be concentrated, typically by ultracentrifugation or antibody-based purification.

Quantitative Assay of Viruses

Two major types of quantitative assays for viruses exist: physical and biological. *Physical* assays, such as hemagglutination, electron microscopic particle counts, optical density measurements, or immunological methods, quantify only the presence of virus particles whether or not the particles are infectious. *Biological* assays, such as the plaque assay or various endpoint methods that have in common the assay of infectivity in cultured cells or *in vivo*, measure only the presence of infectivity and may not count all particles present in a preparation, even many that are in fact infectious. Thus, a clear understanding of the nature and efficiency of both physical and biological quantitative virus assays is required to make effective use of the data obtained from any assay.

Biological Assays

The plaque assay

The plaque assay is the most elegant, the most quantitative, and the most useful biological assay for viruses. Developed originally for the study of bacteriophage by d'Herelle[26] in the early 1900s, the plaque assay was adapted to animal viruses by Dulbecco and Vogt[39] in 1953, an advance that revolutionized animal virology by introducing a methodology that was relatively simple and precisely quantitative, which enabled the cloning of individual genetic variants of a virus and which permitted a qualitative assay for individual virus variants that differ in growth properties or cytopathology.

The plaque assay is based simply on the ability of a single infectious virus particle to give rise to a macroscopic area of cytopathology on an otherwise normal monolayer of cultured cells. Specifically, if a single cell in a monolayer is infected with a single virus particle, new virus resulting from the initial infection can infect surrounding cells, which in turn produce virus that infects additional surrounding cells. Over a period of days (the exact length of time depending on the particular virus), the initial infection thus gives rise through multiple rounds of infection to an area of infection, called a *plaque*. Photomicrographs of plaque foci are shown in Figure 2.6, and stained monolayers containing plaques are shown in Figure 2.7.

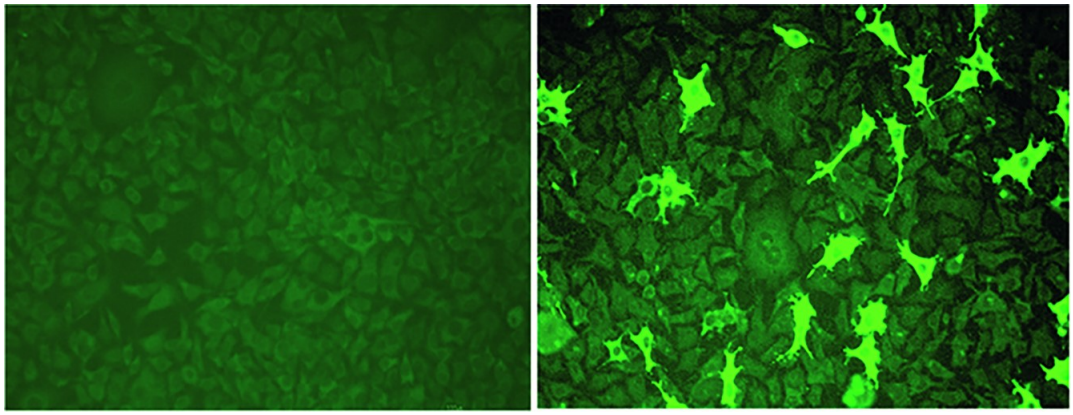

FIGURE 2.6 **Focus of infected cells in a plaque assay.** Mouse L929 cells were infected with serial dilutions of reovirus, and at 30 hours postinfection, cells were fixed and stained with a reovirus-specific antibody and a fluorescently labeled secondary antibody. The number of distinct, clustered stained cells—foci—were counted to quantify viral titer. **Left**, uninfected cells. **Right**, infected cells, zooming in on one focus. (Courtesy of Dr. Andrea Erickson.)

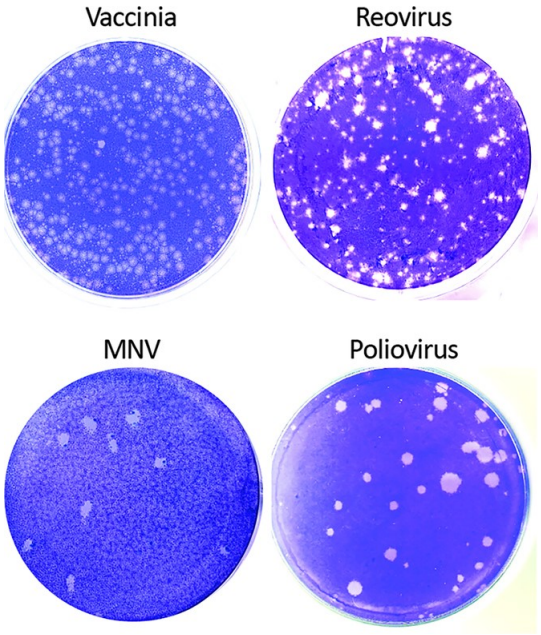

FIGURE 2.7 **Plaque assay.** Monolayers of various cells types were infected with dilutions of the viruses indicated below, and solid or semisolid overlays were added to inhibit diffusion of progeny viruses. After several cycles of replication over several days, overlays were removed, and monolayers were stained with crystal violet dye to visualize plaques. From *left* to *right*, vaccinia virus in BSC40 cells, reovirus in L929 cells, murine norovirus in BV2 cells, and poliovirus in HeLa cells. (Plaque assays images are courtesy of Dr. Don Gammon [vaccinia], Dr. Andrea Erickson [reovirus], Valerie Rodriguez Irizarry [MNV], and Dr. Kristie Ibarra [poliovirus].)

The plaque assay can be used to quantify virus in the following manner. A sample of virus of unknown concentration is serially diluted in an appropriate medium, and measured aliquots of each dilution are seeded onto confluent monolayers of cultured cells. Infected cells are overlayed with a nutrient medium containing an additive to thicken the liquid state into a semisolid, typically agar, agarose, methylcellulose, and more recently the microcrystalline cellulose Avicel. These agents help prevent formation of secondary plaques through diffusion of virus from the original site of infection to new sites, ensuring that each plaque that develops in the assay originated from a single infectious particle in the starting inoculum. After an appropriate period of incubation to allow development of plaques, the monolayer is stained so that the plaques can be visualized. The precise staining technique depends on the cytopathology; however, vital dyes such as neutral red are common. Neutral red is taken up by living cells but not by dead cells; thus, plaques become visible as clear areas on a red monolayer of cells. In cases where the virus

cytopathology results in cell lysis or detachment of cells from the dish, plaques exist literally as holes in the monolayer, and a permanent record of the assay can be made by staining the monolayer with a general stain such as crystal violet. Dishes containing an appropriate number of plaques are counted, and the concentration of infectious virus in the original sample can then be calculated taking into account the serial dilution. The resulting value is called a *titer* and is expressed in plaque-forming units per milliliter (PFU/mL) to emphasize specifically that only viruses capable of forming plaques have been quantified.

A critical benefit of the plaque assay is that it measures infectivity, although it is important to understand that infectivity does not necessarily correspond exactly to the number of virus particles in a preparation. In fact, for most animal viruses, only a fraction of the particles—as few as 1 in 10 to 1 in 10 000—may be infections as judged by comparison of a direct particle count, described later, with a plaque assay. This low *efficiency of plating*, or high particle to infectivity ratio, may have several causes. First, to determine a particle to infectivity ratio, virus must be purified to determine the concentration of physical particles and then subjected to plaque assay. If the purification itself damages particles, the particle to infectivity ratio will be increased. Second, some viruses produce empty particles, or particles that are for other reasons defective during infection, resulting in a high particle to infectivity ratio. Lastly, it is possible that not all infectious particles will form plaques in a given plaque assay. For example, infectious virus may require that cells exist in a specific metabolic state or in a specific stage of the cell cycle; thus, if not all cells in a culture are identical in this regard, only a fraction of the potentially infectious virions may be able to successfully launch an infection and form a plaque.

In addition to its utility as a quantitative assay, the plaque assay also provides a way to detect genetic variants of a virus that possess altered growth properties, and it provides a very convenient method to clone genetically unique variants of a virus. Genetic variants are considered in detail in the Virus Genetics section; in brief, they may comprise viruses that plaque only under certain conditions of temperature or drug treatment, or form plaques of altered size or shape. Because each plaque results from infection with a single infectious virus particle, unique genetic variants of a virus can be cloned simply by picking plaques—that is, literally excising a small plug of semisolid medium and infected cells from a plaque using a Pasteur pipette.

The endpoint method

Viruses that cannot be adapted to either a plaque or a focus assay but nevertheless cause some detectable pathology in cultured cells, embryonated eggs, or animals can be quantified using an endpoint method. Briefly, virus is serially diluted, and multiple replicate samples of each dilution are inoculated into an appropriate assay system. After a suitable incubation period, an absolute judgment is made as to whether or not an infection has taken place. Statistical methods have been devised to calculate the dilution of virus that results in infection in 50% of replicate inoculations, and titers are expressed as the infectious dose 50 (ID_{50}). Assay systems are various and include, for example, observation of cytopathic effect in cultured cells, yielding tissue culture infective dose 50 ($TCID_{50}$); cytopathology or embryonic death in inoculated embryonated chicken eggs, yielding egg infective dose 50 (EID_{50}); or death of an experimental laboratory animal, yielding lethal dose 50 (LD_{50}). As with the plaque assay and the focus assay, the endpoint method has the advantage of measuring infectivity; however, importantly, the unit of infectivity measured by the endpoint method may require more than one infectious particle.

Physical Assays

Direct particle count

The concentration of virus particles in a sample of purified virus can be counted directly using an electron microscope.[102,171] Briefly, a purified preparation of virus is mixed with a known concentration of microscopic marker particles such as latex beads, which can be easily distinguished from virus particles in the electron microscope. Samples of the solution containing virus and beads are then applied to an electron microscope grid and visualized following shadowing or staining. The volume of liquid applied to a given area of the grid can be determined by counting the beads. A solution of virus with a known concentration determined by microscopic particle count, the same solution can be subjected to any number of chemical or spectrophotometric analyses to yield a conversion from protein, nucleic acid, or simply absorbance at a fixed wavelength to a concentration of virus in particles per unit volume. Thus, once a microscopic particle count has been performed, future quantitative assays of purified virus are greatly simplified. Importantly, the direct particle count does not distinguish infectious from noninfectious particles.

Hemagglutination

Many viruses express cell attachment proteins that bind sialic acid, which is abundant on a wide variety of cell types, including erythrocytes. Virions can directly bind erythrocytes, causing them to agglutinate, or form a network of cells and virus, when mixed with virus particles in sufficiently high concentration. Agglutinated erythrocytes can be easily distinguished from cells that are not agglutinated, and thus, hemagglutination can be used as a simple quantitative assay for the presence of a hemagglutinating virus.

In practice, a hemagglutination assay is carried out as follows. Virus is serially diluted, mixed with a fixed concentration of erythrocytes, and the mixture is allowed to settle in a specially designed hemagglutination tray, containing wells with rounded bottoms. Erythrocytes that are not agglutinated are free to roll to the bottom of the well, forming a dense, easily recognizable button or cluster of cells. Erythrocytes that are agglutinated are not free to roll to the bottom of the well but instead evenly coat the bottom surface of the well to form what is called a *shield*. One hemagglutination unit is defined as the minimum amount of virus required to cause agglutination, and the titer of the virus solution, expressed as hemagglutination units per milliliter (HA units/mL), can be calculated taking into account the serial dilution. It is noteworthy that, such as with the direct particle count assay, the hemagglutination assay does not distinguish infectious from noninfectious particles. In addition, because it may require many particles to cause a detectable hemagglutination, one HA unit may represent many physical particles.

Quantitative Considerations in Virus Assay, Cultivation, and Experimentation

Dose-Response in Plaque and Focus Assays

Generally, the number of infectious units observed on a given plate in a plaque assay is a linear function of the dilution of the virus; thus, the development of plaques follows single-hit kinetics, proving that each plaque results from infection with a single virus particle. However, there are exceptions to the “one hit” rule. Viral coinfection can facilitate infection due to genetic processes such as complementation and

recombination, as described in more detail below. Thus, there are cases where a plaque contains two or more parental viruses.[2] Additionally, some viruses require infection with multiple particles to initiate functional infection, including multipartite viruses that package genome segments into separate virions.[94,147] Similarly, murine sarcoma viruses require coinfection with both a defective transforming virus and a nondefective helper virus, in which case the number of foci observed relative to the dilution used follows two-hit kinetics.[60]

Comparison of Quantitative Assays

As noted in the previous individual descriptions, the various quantitative assays of viruses measure different physical and biological properties, and a one-to-one correlation between assays cannot be assumed. Using influenza virus as measured by several different assays provides an example of the magnitude of differences that might be expected in the various assays: Direct particle counting by electron microscopy yields 10^{10} particles/mL, infectivity assay in eggs yields 10^9 egg infectious dose 50 (ID_{50})/mL, infectivity assay by plaque formation yields 10^8 PFU/mL, and hemagglutination assay yields 10^3 hemagglutination units/mL. Hence, relative to a direct particle count, the efficiency of virus detection for this influenza example is 10^{-1} as assayed in eggs, 10^{-2} as assayed in a plaque assay, and 10^{-7} as assayed in a hemagglutination assay. As indicated in the foregoing discussion, some differences result from different properties being measured (eg, physical particles vs infectivity), and some differences result from differences in the sensitivity of the assay (eg, direct particle count vs assay of particles by hemagglutination).

Multiplicity of Infection

Multiplicity of infection, often abbreviated "MOI," measures the average amount of virus added per cell in an infection. Multiplicity of infection can be expressed using any quantitative measure of virus titer—for example, particles/cell, HA units/cell, $TCID_{50}$/cell, or PFU/cell. Because the efficiency of plating varies depending on the method of quantitation used, some knowledge of the infectivity of the sample or the efficiency of plating is required to correctly anticipate the consequences of the use of a particular MOI. The multiplicity of infection used in different protocols can have a profound outcome on the procedure. For example, some viruses, if serially passaged at an MOI of >1 infectious unit/cell, will accumulate spontaneously deleted defective particles that are maintained during passage by the presence of complementing wild-type helper virus.[168] Passage of the same virus at very low MOI (eg, 0.01 infectious units/cell) discourages the accumulation of defective particles because few cells will be coinfected with an infectious and a defective particle, and defective particles cannot replicate in the absence of a wild-type helper. Conversely, other experiments, such as metabolic labeling, assessing the host response to infection, or quantifying the activity of a virally encoded enzyme, are done at high MOI (eg, 10 infectious units/cell) to ensure that all cells in the culture are infected and that the infection is as synchronous as possible. For such experiments, use of too low an MOI may result in an apparently asynchronous infection and a high background owing to the presence of uninfected cells in the culture.

The Poisson distribution can be used to predict the fraction of cells in a population infected with a given number of particles at different multiplicities of infection. As applied to virus infections, the Poisson distribution can be written as:

$$P(k) = e^{-m} m^k / k!$$

where P(k) equals the probability that any cell is infected with k particles, m equals MOI, and k equals the number of particles in a given cell. Sample solutions to the equation are shown in Table 2.2 for commonly used multiplicities of infection.[184] Inspection of this table and consideration of the error inherent in any virus titration involving a serial dilution leads to some significant practical guides in experimental design. Note first that in a culture infected at an MOI of 1 PFU/cell, 37% of cells remain uninfected—an unacceptably high number for an experiment designed to measure a single round of synchronous infection. An MOI of at least 3 is required to infect 95% of the cells in culture. A shortcut is to calculate the fraction uninfected cells by setting k = 0, which reduces the equation to e^{-m}, from which the fraction of cells that are uninfected can be easily calculated. Given that titers can easily be inaccurate by a factor of two, the use of a calculated MOI of 10 ensures that 99% of the cells in a culture will be synchronously infected even if the measured titer is twofold higher than the actual titer.

TABLE 2.2 The Poisson distribution for multiplicity of infection (MOI)

	MOI			
# per cell	1	3	5	10
0	0.37	0.05	0.01	0.00
1	0.37	0.15	0.03	0.00
2	0.18	0.22	0.08	0.00
3	0.06	0.22	0.14	0.01
4	0.02	0.17	0.18	0.02
5	0.00	0.10	0.18	0.04
6	0.00	0.05	0.15	0.06
7	0.00	0.02	0.10	0.09
8	0.00	0.00	0.07	0.11
9	0.00	0.00	0.04	0.13
10	0.00	0.00	0.02	0.13

One-Step Growth Experiment

The one-step growth experiment is a classic assay developed initially for bacteriophage[40] and still frequently used to determine the essential growth properties of a virus. The goal of this experiment is to measure the time course of virus replication and the yield of virus per cell during a single round of infection. The experiment is carried out as follows. Several dishes containing confluent monolayers of an appropriate cultured cell are infected simultaneously with virus at a high MOI (eg, 10 PFU/cell). After an adsorption period, monolayers are washed to remove unabsorbed virus and then incubated in culture medium. At various times after infection, virus from individual dishes is harvested, and at the completion of the experiment, the virus titer in samples representing each time point is determined. The virus yield at each point can be converted to PFU/cell (also called *burst size*) by dividing the total amount of virus present in the sample by the number of cells originally infected in the sample. Two examples of one-step growth experiments with coxsackievirus B3 or Rio Bravo virus are shown in Figure 2.8. Several features of the growth curve are noteworthy. First, during the first several hours of the infection, the titer in the cultures decreases and then increases. This initial dip in the growth curve is called *eclipse* and results from the fact that early during the experiment, virus attached to the cell surface but not uncoated remains infectious; however, infectivity is lost following uncoating during the first few hours of infection, and infectivity is recovered only after new virus is produced. The infection then enters a rapid growth phase, followed by a plateau. The plateau results from the fact that all infected cells have reached the maximum yield of virus, or have died or lysed, depending on the type of virus infection. The time interval from infection to plateau represents the time required for a single cycle of growth, and the yield of virus at plateau shows the amount of virus produced per cell. The experiment in Figure 2.8 suggests that coxsackievirus B3 has a relatively fast replication cycle, with progeny viruses detectable at between 5 and 7 hours postinfection. Conversely, Rio Bravo virus is not detected until 16 hours. One-step growth experiments to determine the length of a single replication cycle are ideally performed in the target cell of interest, as replication dynamics can differ between cell types, particularly depending on whether cell intrinsic antiviral pathways are intact.

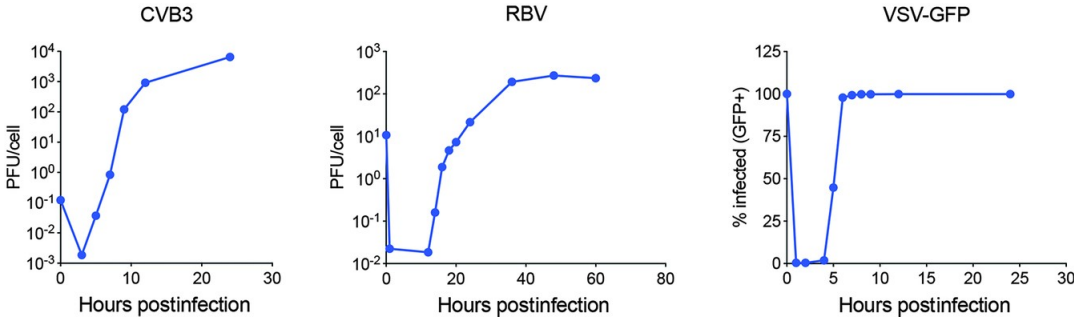

FIGURE 2.8 **One-step viral growth experiments. Left**. Caco-2 cells were infected with coxsackievirus B3 at an MOI of 0.1, and progeny viruses were collected at a variety of time points followed by plating dilutions for plaque assays on HeLa cells. Data are presented as plaque forming units (PFU) per cell. (Courtesy of Dr. Yao Wang.) **Middle**. Similar to above, Huh7.5 cells were infected with Rio Bravo virus at an MOI of 5, and progeny viruses were titered on BHK cells. (Courtesy of Dr. Ian Boys.) **Right**. Human *STAT1*$^{-/-}$ fibroblasts were infected with VSV-GFP at an MOI of 5, and serially collected supernatants containing progeny viruses were used undiluted to re-infect naive cells. Intact cells were harvested, fixed with paraformaldehyde, and the percentage of infected (GFP+) cells was quantified by flow cytometry.

Multiplicity of infection is a critical factor in the design of a virus growth experiment. A true one-step growth experiment can only be done at high MOI. If the MOI is too low and a large fraction of cells are left uninfected, then virus produced during the first round of infection will replicate on previously uninfected cells, and thus multiple rounds of infection rather than one round will be measured. A growth experiment done at low MOI has utility in that it measures both growth and spread of a virus in culture; however, the time from infection to plateau does not accurately reflect the time required for a single cycle of infection. It is also noteworthy that some mutant phenotypes are multiplicity dependent.[11]

Modern Viral Assays and Approaches

As with all fields of science, virology has modernized significantly over the past couple of decades. Whereas classical virology has often focused on the virus side of "virus-host interactions," modern approaches are increasingly considering the host side, including a detailed examination of host factors required for viral replication, as well as host responses to viral infection, in particular the immune response. This chapter primarily emphasizes approaches to study virology that are more consistent with classical themes in the field, for example, understanding viral gene function, viral replication kinetics, and virus evolution. However, we also briefly discuss modern approaches that are being used to complement classical virology on the virus side and to augment our understanding of virus-host interactions from the perspective of the host.

Viruses Expressing Foreign Reporter Genes

Genetic approaches have allowed many viruses to be modified to express a variety of reporter genes from the viral genome. Historically, these recombinant viruses expressed foreign genes such as chloramphenicol acetyltransferase or β-galactosidase, which could be monitored by enzyme assay and used as surrogates for viral gene expression. These strategies have largely been supplanted by fluorescent or bioluminescent reporters such as green fluorescent protein (GFP) and firefly luciferase, respectively.

Reporter viruses have distinct advantages and limitations relative to their nonreporter parental viruses. Viruses expressing fluorescent reporters such as GFP can be used in cell culture to quantify viral infectivity, that is, the percentage of cells that become infected. Infectivity is typically quantified by flow cytometry or fluorescence microscopy and can then be used to calculate viral titers based on Poisson distribution. Titers can be expressed as "fluorescent cell units," which is analogous to the plaque-forming units derived from plaque assays. However, the timing of viral replication needs to be considered and restricted to the first replication cycle, as viral spread will lead to more GFP-positive cells and an inaccurate assessment of true infectivity. Infectivity, as reflected by GFP positivity, can be used in many other applications. For example, a one-step growth experiment can be performed as described above, with the difference being that progeny viruses are detected by re-infecting naive cells and assessing infectivity by flow cytometry. An example is shown in Figure 2.8 with VSV-GFP. These data indicate that a single replication

cycle is between 5 and 6 hours. In this experiment, supernatants containing progeny viruses were not diluted as is standard in a plaque assay. Thus, this type of one-step growth curve can only determine the timing of a replication cycle, but it does not give information on how many infectious units of virus are produced per cell. This latter metric could be obtained if supernatants were serially diluted, and the data were back-calculated based on the Poisson distribution.

Infectivity can simultaneously be complemented with quantitation of GFP mean fluorescence intensity (MFI). Depending on the virus, GFP MFI can provide distinct information about the viral infection process. For example, many GFP-expressing RNA viruses express the reporter from the viral genome similar to other viral proteins. Thus, GFP serves as a surrogate for viral protein synthesis, and the effect of any perturbation (eg, drug treatment, genetic modification, etc.) can be quantified by GFP MFI. This also holds for viruses expressing luciferase; indeed, many drug screening efforts now rely on viral genomes expressing luciferase for high-throughput assays (discussed below). For DNA viruses with highly defined temporal gene expression patterns, such as herpesviruses or adenoviruses, GFP can inserted into specific regions of the genome and used as a surrogate to monitor the temporal dynamics of viral gene expression. In other genetic strategies, GFP can be fused to a viral protein, and provided that the fusion does not disrupt viral protein function, the expression, subcellular localization, and dynamics of the viral protein can be monitored with a variety of microscopic techniques. Both fluorescent and bioluminescent reporters can additionally be used to monitor viral dynamics *in vivo*. For example, a virus expressing firefly luciferase can be administered to a mouse, and after a defined amount of time, the animal can be injected with luciferin substrate and imaged to assess which organs and tissues are productively infected (Fig. 2.9).

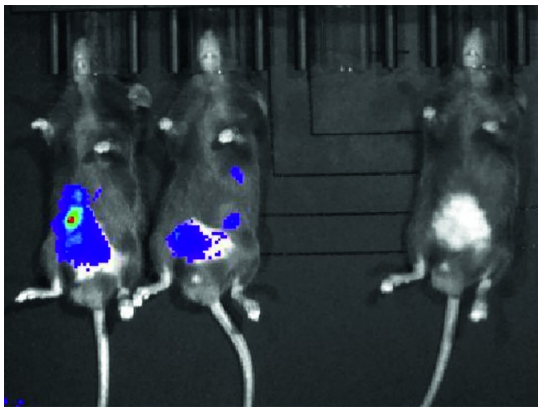

FIGURE 2.9 ***In vivo* bioluminescence imaging**. Mice were mock-infected (*right*) or infected with 10^6 PFU with gammaherpesvirus MHV68 expressing firefly luciferase under control of the M3 promoter (*left*). To quantitate luciferase activity, mice were injected intraperitoneally with luciferin and imaged within 20 minutes of injection. (Courtesy of Dr. Tiffany A. Reese.)

Reporter viruses have important limitations. Some viruses may not tolerate large foreign gene inserts due to packaging limits, negative effects on replication, or disruption of important secondary structural features in the viral genome. If the reporter gene is successfully inserted, the reporter virus is frequently attenuated relative to the parent virus. Additionally, the reporter gene may not remain stable over time. This is particularly true with +ssRNA viruses expressing foreign genes. After serially passaging, and in some cases after even a single passage, the foreign insert is lost, likely due to a recombination event that reverts the virus to "wild type." Thus, generating stocks from passaged virus is usually a suboptimal approach. Summarily, while reporter viruses are powerful tools, they have several limitations, which must be considered during experimental design.

High-Throughput Approaches to Examine Virus-Host Interactions

The field of virology has been highly amenable to advances in high-throughput approaches. Key aspects of high-throughput approaches include the ability to assay hundreds, thousands, or even tens of thousands of samples in a relatively short time frame. This is accomplished by sophisticated instrumentation such as luminometers, flow cytometers, or high-content microscopes, each of which can handle multiwell plates (eg, 96-, 384-, or 1536-well plates). Early examples of high-throughput approaches in virology include screening of a 100 000-compound library for inhibitors of SARS-CoV using cell viability as the readout.[145] Many high-throughput approaches are limited, because they can only provide readouts at a single time point. For example, a drug screen that quantifies protection from virus-induced cell killing will typically be performed at a single time point that has been predetermined using known controls. Similarly, a high-throughput screen for antibodies that inhibit infection by a GFP-expressing virus will usually use a single infection time point for the final readout. However, newer live cell imaging and analysis platforms (eg, Incucyte) allow for real-time quantitative live cell imaging, thus providing finely resolved temporal resolution of viral replication and/or virus-induced changes to the host cell.

Rapid technological advances and decreasing costs of nucleic acid synthesis have revolutionized high-throughput genetic screens across all areas of biology, including virology. After the discovery of RNA interference in *Caenorhabditis elegans*, gene silencing technology based on small interfering RNA (siRNA) quickly became the method of choice for silencing genes in mammalian cells. Targeted and genome-scale siRNA libraries were developed and screened in cell-based assays to identify host factors that were required for viral replication. The first genome-scale screens were performed with HIV-1,[9,92,183] with many other viruses to follow. These screens provided a wealth of new information about the specific host proteins required for infection and, in some cases, host factors that contributed to cell intrinsic control of viral replication. However, screening hits from lab to lab did not have extensive overlap,[10] raising concerns about the utility of this method. Similar to RNAi/siRNA technology, the CRISPR antiviral pathway in prokaryotes has also been co-opted to modulate gene expression in eukaryotic cells. CRISPR-based strategies can be used to (1) knock genes out by targeting DNA at the gene of interest, (2) reduce gene expression by targeting mRNA, or (3) induce gene expression by promoting gene-specific transcription. To date, the field of virology has gained the most traction with genome-scale CRISPR knockout libraries, which have been used to identify with high confidence and reproducibility host factors required for multiple viruses, including flaviviruses,[104] HIV-1,[122] influenza A virus,[59] and alphaviruses.[182] Conversely, several genome-scale CRISPR-based screens have also been used to identify inhibitory host factors targeting viruses such as influenza A virus, yellow fever virus, and murine norovirus.[66,120,135] Given the low cost and relative ease of performing CRISPR screens, this technology is certain to continue revealing host genes with prominent roles during virus-host interactions.

Virus Genetics

Viruses are subject to the same genetic principles at work in other living systems, namely mutation, selection, complementation, and recombination. Genetics impacts all aspects of virology, including the natural evolution of viruses, clinical management of infections, and experimental virology. For example, antigenic variation, which is a direct result of mutation and selection, plays a prominent role in the epidemiology of influenza virus and HIV in the human population, and mutation to drug resistance offers a significant challenge to the clinical management of virus infections with antiviral drugs. This section deals primarily with the application of experimental genetic techniques to basic virology.

A major goal of experimental virology is to understand the functional organization of a viral genome. This means determination of the structure of a virus genome at the nucleotide sequence level, coupled with isolation and characterization of mutant viruses. Thus, genetic analysis of viruses is of fundamental importance to experimental virology.

Before the advent of modern nucleic acid technology—that is, during a *classical* period of *forward* genetics—genetic analysis of viruses consisted of the random, brute force isolation of large numbers of individual virus mutants, followed first by complementation analysis to determine groupings of individual mutants into genes, then recombination analysis to determine the physical order of genes on the virus genome, and finally the phenotypic analysis of mutants to determine gene function. This approach, pioneered in the 1940s through the 1960s in elegant studies of several bacteriophage, was the primary method for identifying, mapping, and characterizing virus genes. The application of cell culture techniques to animal virology opened the door to classical genetic analysis of animal viruses, resulting in a flurry of activity in the 1950s through the 1970s, during which time hundreds of mutants were isolated and analyzed in prototypical members of most of the major animal virus families.[51] Nucleic acid technology introduced in the 1970s brought with it a variety of techniques for physical mapping of genomes and mutants, including restriction enzyme mapping, marker rescue, and DNA sequence analysis, which together replaced recombination analysis as an analytic tool. Mutants and techniques from the classical period continue to be of enormous utility today; however, recombinant DNA technology has brought with it *reverse* genetics, in which the structure of the genome is determined first using entirely physical methods, then the function of individual genetic elements is determined by analyzing mutants constructed in a highly targeted fashion. Furthermore, modern low-cost sequencing technology now means that thousands of complete viral genomes can be generated daily. However, additional studies are still required to understand genome structure-function.

The genetic approach to experimental virology has the profound advantage of asking of the organism under study only the most basic question—What genes are needed to survive and why?—without imposing any further bias or assumptions on the system. Organisms often respond with surprises that the most ingenious biochemist or molecular biologist would never have imagined. What follows is a summary of the critical elements of both the classical and modern approaches to virus genetics as applied to experimental virology.

Fundamental Genetic Concepts

Concepts fundamental to genetic analysis of other organisms apply to genetic analysis of viruses, and a clear understanding of these concepts is essential. The most important of these concepts are briefly summarized next.

Wild Type, Mutations, and Mutants

Wild type refers to a genotype or phenotype that predominates in nature. It is important to understand that in the context of experimental virus genetics, a virus designated as wild type can differ significantly from the virus that actually occurs in nature. For example, virus genetics often relies heavily on growth and assay of viruses in cell culture, and natural isolates of viruses may undergo significant genetic change during adaptation to cell culture. In addition, viruses to be designated as wild type should be plaque purified before initiating a genetic study to ensure a unique genetic background for mutational analysis. *Mutation* refers to a sequence alteration in the nucleic acid genome of a virus, whereas viruses containing mutation(s) in their genomes are referred to as *mutants*.

Genotype and Phenotype

Genotype refers to the actual genetic change from wild type in a particular virus mutant, whereas *phenotype* refers to the measurable manifestation of that change. This distinction is emphasized by the fact that a single genotype may express different phenotypes depending on the assay. Thus, for example, the same missense mutation in a virus gene may cause temperature sensitivity in one cell line but not another, or a deletion in another virus gene may have no effect on the replication of virus in culture but may alter virulence in an animal model.

Selection and Screen

Selection and screen refer to two fundamentally different methods of identifying individual virus variants contained in a mixed population of viruses. *Selection* implies that a condition exists where only the desired virus will grow, and growth of unwanted viruses is suppressed. Thus, a drug-resistant virus can be identified by plating a mixture of wild-type, drug-sensitive, and mutant, drug-resistant viruses together on the same cell monolayer in the presence of the inhibitory drug, thereby selecting for drug-resistant viruses that grow and against wild-type viruses that do not grow. A *screen* implies that both the desired virus variant and one or several other unwanted virus types grow under a given condition, such that many viruses must be analyzed individually to identify the desired variant. For example, in searching for a temperature-sensitive mutant (ie, a virus whose growth is inhibited relative to wild-type virus at an elevated temperature), no condition exists under which the mutant alone will grow. Therefore, virus must be plated at a low temperature where both wild-type and mutant virus will grow, and plaques tested individually for temperature sensitivity. Sometimes a screen can be streamlined by introducing a phenotypic marker into the variant of choice. For example, a knockout virus might be constructed by inserting reporter genes such as β-galactosidase or GFP into the virus gene to be inactivated. Thus, viruses containing the insertional knockout can be distinguished from unmodified viruses.[180] This latter example is still a screen, because both wild-type and mutant viruses grow under the conditions used; however, the screen is simplified because mutant viruses can be readily identified by their color, obviating the need to pick and test individual plaques. Selections have considerable advantages over screens but are not always possible.

Essential and Nonessential

The terms *essential* and *nonessential* describe phenotypes, specifically whether a given gene is required for growth under a specific condition. Most viruses are finely tuned through selection to fit a specific niche. In some cases, not all viral genes are absolutely required for virus replication in that niche, particularly for viruses with large genomes. Furthermore, if the niche is changed—such as from a natural animal host to a cell line in a laboratory—some genes that may have been essential for productive infection in the animal may not be required for replication in cell culture. Genes that are required for growth under a specific condition are termed *essential*, and those that are not required are termed *nonessential*. Because as a phenotype essentiality may be a function of the specific test conditions, the test conditions need to be specified in describing the mutation. As an example, the herpesvirus thymidine kinase gene is nonessential for virus replication in cell culture. Genes that are either essential or nonessential under a given condition present unique characteristics for analysis. Thus, mutants in nonessential genes may be easy to isolate because the gene can be deleted, although the function of the gene may be difficult to determine because, by definition, nonessential genes have no phenotype. Conversely, genes that are essential can be used to study gene function by characterizing the precise replication defect caused by a mutation in the gene; however, acquiring the appropriate mutant is confounded by the necessity for identifying a condition that will permit growth of the virus for study. Generally, viruses with small genomes have few if any nonessential genes. For example, poliovirus encodes just 11 proteins, and all are essential for complete viral replication in cultured cells.

Loss of Function and Gain of Function

Mutations that alter the function of a gene can be subclassified into two categories, loss of function or gain of function. Both loss-of-function and gain-of-function mutations are useful tools in virology. For *loss-of-function* mutations, the gene product lacks a molecular function of the wild-type gene. The inactivation of the viral gene product may be partial or complete. Examples of loss-of-function mutations include lack of vaccinia virus replication in human cells from K1L gene deletion,[124] and lack of herpes simplex virus latency reactivation from viral thymidine kinase gene deletion.[16] Conversely, for *gain-of-function* mutations, the gene product's effect gets stronger or may even acquire different or abnormal functions. Examples of gain-of-function mutations include point mutations in influenza virus HA protein that increase transmission[67,74] and a coxsackievirus capsid mutation that increases viral replication speed in cultured cells.[96] Notably, there are often fitness "tradeoffs" for gain-of-function mutants, where fitness gain in one environment confers fitness loss in a different environment.[35]

Study of Mutations and Mutants

Spontaneous Mutation

Spontaneous mutation rates in viruses are measured by fluctuation analysis,[77] a technique pioneered by Luria and Delbruck[101] for analysis of mutation in bacteria, and later adapted to viruses by Luria.[99] Fluctuation analysis consists of measuring the proportion of spontaneous mutants with a particular phenotype in many replicate cultures of virus and applying the Poisson distribution to these data to calculate a mutation rate.

Both DNA and RNA viruses undergo spontaneous mutation; however, the spontaneous mutation rate in RNA viruses is usually much higher than in DNA viruses. In general, the mutation rate at a specific site in different DNA viruses ranges from 10^{-8} to 10^{-11} per replication, whereas in RNA viruses, it is at least hundred-fold higher, between 10^{-3} and 10^{-6} per replication. The difference in mutation rate observed between RNA and DNA viruses is primarily from differences in the replication enzymes. Specifically, the DNA-dependent DNA polymerases used by DNA viruses contain a proofreading function, whereas the reverse transcriptases used by retroviruses and RNA-dependent RNA polymerases used by RNA viruses lack a proofreading function. The difference in spontaneous mutation rate has profound consequences for both the biology of the viruses and for laboratory genetic analysis of viruses. Specifically, RNA viruses exist in nature as *quasispecies*[34]—that is, populations of virus variants in relative equilibrium with the environment but capable of swift adaptation owing to a high spontaneous mutation rate. Conversely, DNA viruses are genetically more stable but less adaptable. In the laboratory, the high mutation rate in RNA viruses presents difficulties in routine genetic analysis because mutants easily revert to wild-type virus that can outgrow the mutant virus.

It is noteworthy that whereas the actual mutation rate at a single locus is probably relatively constant for a given virus, the *apparent* mutation rate to a given phenotype depends on the nature of the mutation(s), which can give rise to that phenotype. For example, spontaneous mutation to bromodeoxyuridine (BrdU) resistance in vaccinia virus may occur at least 10-100 times more frequently than spontaneous reversions of temperature-sensitive mutations to a wild-type, temperature-insensitive phenotype. In the case of BrdU resistance, any mutation that inactivates the thymidine kinase causes resistance to BrdU, and thus, there are literally hundreds of different ways in which spontaneous mutation can give rise to BrdU resistance. By contrast, a temperature-sensitive mutation is usually a single-base missense mutation, in which may exist only one possible mutational event that could cause reversion to the wild-type phenotype; thus, the apparent spontaneous mutation rate for the revertant phenotype is lower than the apparent spontaneous mutation rate to the BrdU-resistant phenotype. From a practical perspective, the apparent spontaneous mutation rate for specific selectable phenotypes may be sufficiently high such that induction of mutants is unnecessary for their isolation. However, for mutants where the desired mutational events are rare and a screen must be used rather than a selection (eg, temperature-sensitive mutants), induced mutation may be required for efficient isolation of mutants. Induced mutation is particularly useful for viruses with low error rates, such as many DNA viruses.

Induced Mutation

While many RNA viruses have mutation frequencies high enough for relatively straightforward isolation of viral mutants, the incidence of spontaneous mutations in many DNA viruses is low enough that induction of mutation is a practical prerequisite. It is usually desirable to induce limited single-base changes, and for this purpose, chemical mutagens are most appropriate. Commonly used chemical mutagens are of two types: *in vitro* mutagens and *in vivo* mutagens.[36] *In vitro mutagens* work by chemically altering nucleic acid and can be applied by treating virions in the absence of replication. Examples of *in vitro* mutagens include hydroxylamine, nitrous acid, and alkylating agents, which through chemical modification of specific bases cause mispairing leading to missense mutations. *In vivo* chemical mutagens comprise compounds such as nucleoside analogs that must be incorporated during viral replication and thus must be applied to an infected cell. One of the most effective mutagens is the alkylating agent nitrosoguanidine, which although is capable of alkylating nucleic acid *in vitro* is most effective when used *in vivo*, where it works by alkylating guanine residues at the replication fork, ultimately causing mispairing. The effectiveness of a mutagenesis is often assayed by observing the killing effect of the mutagen on the virus. Overall, the use of mutagens can increase the mutation frequency several hundred-fold, such that desired mutants may comprise as much as 0.5% of the total virus population.

RNA viruses have relatively high mutation frequencies, and increasing their mutation frequencies even more with mutagen treatment can

extinguish populations through "lethal mutagenesis." For example, poliovirus propagated in the presence of the mutagenic nucleoside analog ribavirin has an increased mutation frequency, which can dramatically reduce viral fitness.[25] Using mutagenic nucleoside analogs to induce lethal mutations in RNA viruses has been applied to several systems, ranging from HIV to coronaviruses.[123,150]

Double Mutants and Siblings

The existence of *double mutants* and *siblings* can theoretically complicate genetic analysis of a virus. A double (or multiple) mutant is defined as a virus that contains more than one mutation contributing to a phenotype. Theoretically, because the probability that a double mutant will be created increases as the dose of a mutagen is increased, there is a practical limit to the amount of induced mutation that is desirable. Double mutants are usually revealed as mutants that are noncomplementing with more than one mutant or are impossible to map by recombination or physical methods. Siblings result from replication of mutant virus either through amplification of a mutagenized stock or during an *in vivo* mutagenesis. The only completely reliable method to avoid isolation of sibling mutants is to isolate each mutant from an independently plaque-purified stock of wild-type virus.

Mutant Genotypes

There exist two basic categories of mutation: base substitution and deletion/insertion mutations. Both mutation types can occur with consequence in either a protein coding sequence or in a control sequence, such as a transcriptional promoter, a replication origin, or a packaging sequence. *Base substitution* mutations consist of the precise replacement of one nucleotide with a different nucleotide. In coding sequences, base substitution mutations can be silent, causing no change in amino acid sequence of a protein; they can be missense, causing replacement of the wild-type amino acid with a different residue; or they can be nonsense, causing premature translation termination during protein synthesis. *Deletion and insertion* mutations comprise deletion or insertion of one or more nucleotides in a nucleic acid sequence. In a coding sequence, deletion or insertion of multiples of three nucleotides can result in precise deletion or insertion of one or more amino acids in a protein sequence. In a coding sequence, deletions or insertions that do not involve multiples of three nucleotides result in a shift in the translational reading frame, which almost invariably results in premature termination at some distance downstream of the mutation. In general, nonsense mutations, frame shift mutations, or large in-frame insertions or deletions are expected to inactivate a gene, whereas missense mutations may cause inactivation or much more subtle phenotypes such as drug resistance or temperature sensitivity.

Mutant Phenotypes

In the context of experimental virology where a goal is to understand the function of individual virus genes, mutants that inhibit virus replication by inactivating a viral gene are useful. The nonproductive infections with these lethal mutants can be studied in detail to determine the precise aspect of virus replication that has been affected, thus providing information about the normal function of the affected gene. However, one must be able to grow the mutant to conduct experiments. Thus, a condition must be found where the mutation in question is not lethal—hence, the general class of mutant phenotypes, *conditional lethal*. Conditional lethal mutants are one of the most useful classes of mutant phenotypes, consisting of host-range, nonsense, temperature-sensitive, and drug-dependent phenotypes, described individually in the next section. Two additional classes of mutant phenotypes—resistance and plaque morphology—have very specific application to genetic analysis of viruses and are also described.

Host range

A host-range virus mutant is broadly defined as a mutant that grows on one cell type and not on another, in contrast to wild-type virus, which grows on both cell types. Two general subcategories of host-range mutants exist: natural and engineered. *Natural* host-range virus mutants are relatively rare, primarily because they must be identified by brute force screen or serendipity. The existence of a host-range phenotype implies that a specific virus-host interaction is compromised, which also implies that for any specific host-range phenotype, only one or a limited number of virus genes will be targeted. A classic example of a natural host-range mutant is the host range-transformation (hr-t) mutants of mouse polyoma virus, which affect both small and middle T antigens and grow on primary mouse cells but not continuous mouse 3T3 cell lines.[5] *Engineered* host-range mutants are constructed by deleting an essential gene of interest in the virus while at the same time creating a cell line that expresses the gene. The engineered cell line provides a permissive host for growth of the mutant virus because it complements the missing virus function, whereas the normal host lacking the gene of interest provides a nonpermissive host for study of the phenotype of the virus. This technology has been useful for study of a variety of viruses, notably adenovirus and herpes simplex virus, where it has facilitated study of several essential virus genes.[29,79]

Nonsense mutants

Nonsense mutants contain a premature translation termination mutation in the coding region of the mutant gene. They are formally a specific class of conditionally lethal, host-range mutants. Specifically, the permissive host is one that expresses a transfer RNA (tRNA) containing an anticodon mutation that results in insertion of an amino acid in response to a nonsense codon, thus restoring synthesis of a full-length polypeptide and suppressing the effects of the virus nonsense mutation. The nonpermissive host is a normal cell in which a truncated, nonfunctional polypeptide is made. In practice, most nonsense mutants in existence have been isolated by random mutagenesis followed by a brute force screen for host range. Nonsense mutants have three distinct advantages for the conduct of virus genetics: (1) mutants can be isolated in virtually any essential virus gene using one set of permissive and nonpermissive hosts and one set of techniques; (2) the mutations result in synthesis of a truncated polypeptide, thereby facilitating identification of the affected gene; and (3) virus mutants can be engineered relatively easily because the exact sequence of the desired mutation is predictable. Nonsense mutants have provided the single most powerful genetic tool in the study of bacteriophage, where efficient, viable nonsense suppressing bacteria are readily available. Unfortunately, attempts to isolate nonsense-suppressing mammalian cells have met with only limited success, probably because the nonsense-suppressing tRNAs are lethal in the eukaryotic host.[118,144]

Temperature sensitivity

Temperature sensitivity is a type of conditional lethality in which mutants can grow at a low temperature but not a high temperature, in contrast to wild-type virus, which grows at both temperatures. Genotypically, temperature-sensitive mutations result usually from relatively subtle single

amino acid substitutions that render the target protein unstable and hence nonfunctional at an elevated or nonpermissive temperature while leaving the protein stable and functional at a low, permissive temperature. Additionally, mutations in noncoding regions can confer temperature sensitivity, likely through effects on RNA structure.[140] Temperature-sensitive mutants are usually isolated by random mutagenesis followed by brute force screening for growth at two temperatures. Screening can be streamlined by a plaque enlargement technique in which mutagenized virus is first plated at a permissive temperature, then stained and shifted to a nonpermissive temperature after marking the size of plaques, to screen for plaques that do not increase in size at the nonpermissive temperature.[149] Replica plating techniques that permit relatively straightforward screening of thousands of mutant candidates in yeast and bacteria have not been successfully adapted to virology; thus, a screen for temperature sensitivity, even when streamlined with plaque enlargement, ultimately depends on the laborious but reliable process of picking and testing individual plaques. Temperature-sensitive mutants have the profound advantage of theoretically accessing any essential virus gene using a single set of protocols. Temperature-sensitive mutants have proved enormously useful in all branches of virology but have been particularly useful for the study of animal viruses, where nonsense suppression has not been a viable option. Cold-sensitive mutants (ie, mutants that grow at a high but not a low temperature) comprise a relatively rare but nevertheless useful alternate type of temperature-sensitive mutants.

Drug resistance and dependence

Several antiviral compounds have been identified, and virus mutants that are resistant to or depend on these compounds have been useful in genetic analysis of viruses. A few compounds have been identified that target similar enzymes in different viruses, including phosphonoacetic acid, which inhibits DNA polymerases,[64,152] and BrdU, which targets thymidine kinases.[38,156] More often, however, antiviral drugs are highly specific for a gene product of one particular virus—for example, guanidine, which targets the polio 2C NTPase[128]; acyclovir, which targets the herpes simplex virus thymidine kinase and DNA polymerase[17,143]; amantadine, which targets the influenza virus M2 virion integral membrane ion channel protein[63]; or isatin-β-thiosemicarbazone, which is highly specific for poxviruses and targets at least two genes involved in viral transcription.[19,24] The most useful drugs are those that inhibit wild-type virus growth in a plaque assay without killing cells in a monolayer, such that resistant or dependent viruses can be selected by virtue of their ability to form plaques on a drug-treated monolayer.

Drug-resistant or drug-dependent virus mutants have two general uses in virus genetics. First, they can be useful in identifying the target or mechanism of action of an antiviral drug. For example, studies of influenza virus mutants resistant to amantadine were of importance in characterizing both the *M2* gene and the mechanism of action of amantadine.[129] Similarly, viruses resistant to the mutagenic nucleoside analog ribavirin have polymerase mutations that increase fidelity of RNA virus replication, further supporting viral mutagenesis as an antiviral mechanism.[25,126] Second, resistant or dependent mutants provide selectable markers for use in recombination mapping, for the assessment of specific genetic protocols, or for selection of recombinant viruses in reverse genetic protocols. For example, guanidine resistance has been used as a marker for use in three-factor crosses in recombination mapping of poliovirus temperature-sensitive mutants[23]; phosphonoacetic acid resistance and isatin-β-thiosemicarbazone dependence have been used in vaccinia virus to assess the efficiency of marker rescue protocols,[43] and acyclovir resistance and BrdU resistance, resulting from mutation of the herpesvirus or poxvirus thymidine kinase genes, have been used in both herpesviruses and in poxviruses to select for insertion of engineered genes into the viral genome.[18,105,121]

Plaque morphology

Plaque morphology mutants are those in which the appearance of mutant plaques is readily distinguishable from wild-type plaques. Most commonly, the morphological distinction is plaque size (ie, mutant plaques may be larger or smaller than wild-type plaques); however, other morphological distinctions are possible, such as formation of clear vs turbid bacteriophage plaques. Most plaque morphology mutants affect very specific virus functions, which in turn affect the virus-host relationship in a fashion that impacts on the appearance of a plaque. Notable examples from bacteriophage research include clear plaque mutants of bacteriophage lambda and rapid lysis mutants of the T-even bacteriophage. Wild-type lambda forms turbid plaques because some percentage of cells are lysogenized and thus survive the infection, leaving intact bacteria within a plaque. Clear mutants of lambda typically affect the lambda repressor such that lysogeny is prevented and all infected bacteria lyse, resulting in a clear plaque.[81] Wild-type T-even phages produce small plaques with a turbid halo because only a fraction of infected bacteria lyse during a normal infection, a phenomenon called *lysis inhibition*. Rapid lysis mutants, which affect a phage membrane protein, do not display lysis inhibition and as a result form large, clear plaques.[68] Examples from animal virus research include large plaque mutants of adenovirus and coxsackievirus, and syncytial mutants of herpes simplex virus. The large plaque phenotype in adenovirus results from faster than normal release of virus from infected cells,[86] and the large plaque phenotype in coxsackievirus results from reduced binding to inhibitory glycans.[170] Syncytial mutants of herpesvirus express altered virus surface glycoproteins and result in fusion of infected cells, whereas wild-type virus infection causes cells to round and clump without significant fusion. Thus, syncytial mutants form large plaques readily distinguishable from the smaller dense foci caused by wild-type virus.[137] All of these specific plaque morphology mutants have value either in the study of the actual functions affected or as specific phenotypic markers for use in recombination studies, where they can be used in the same fashion as drug resistance markers.

In addition to the existence of specific plaque morphology loci in several viruses, it is noteworthy that any mutation that affects virus yield, growth rate, or spread may result in production of a smaller than wild-type plaque, which can be useful in genetic experiments. Thus, many temperature-sensitive mutants form smaller than wild-type plaques even at the permissive temperature because the mutant gene may not be fully functional even under permissive conditions, and this property is often useful in mutant isolation or for distinguishing wild-type from mutant virus in plaque assays involving several virus variants. Finally, unique functions of specific viral proteins can alter plaque morphology. Vaccinia virus encodes two proteins, A33 and A36, that are expressed on the cell surface early after infection and “mark” the cell as infected.[31] As a consequence, superinfecting virions are repelled and available to infect other uninfected cells, enabling spread. Mutant viruses lacking expression of these proteins have small plaque phenotypes.

Neutralization escape

Neutralization escape mutants are a specific class of mutants selected as variant viruses that form plaques in the presence of neutralizing antibodies. Such mutants affect the structure or modification of viral surface proteins and have been of value in studies of virus structure, antigenic variation, and virus-cell interactions.[55,70] Neutralization escape has been particularly well studied during the SARS-CoV-2 pandemic given its practical relevance for immune evasion and spread of viral variants.[172]

Reversion

Reversion may be defined as mutation that results in a change from a mutant genotype to the original wild-type genotype. Accordingly, revertants in a stock of mutant virus are revealed as viruses that have acquired a wild-type phenotype. Spontaneous reversion of missense mutations probably results from misincorporation during replication, because the reversion frequency of different viruses often reflects the error rate of the replication enzyme. Spontaneous reversion of significant deletion mutations occurs rarely, if at all, because reversion would require replacement of missing nucleotides with the correct sequence. Reversion impacts on viral genetics in two ways. First, in any genetic experiment involving mixed infections with two genetically different viruses, wild-type viruses can arise either through reversion or recombination; in most cases, it is important to be able to distinguish between these two processes. This is discussed in more detail in the later sections describing complementation and recombination. Second, as described earlier in the description of spontaneous mutation, if the spontaneous reversion rate is extremely high, revertants can easily come to dominate a mutant virus stock, thus obscuring the mutant phenotype and causing serious difficulties in both genetic and biochemical analysis of mutants.

Leakiness

Not all conditionally lethal mutants are completely defective in replication under nonpermissive conditions, and leakiness is a quantitative measure of the ability of a mutant virus to grow under nonpermissive conditions. Leakiness can be quantified with a one-step growth experiment. To quantify leakiness of a temperature-sensitive mutant, for example, cells are infected at a high MOI with wild-type or mutant virus, infected cells are incubated at either permissive or nonpermissive temperatures, and maximum virus yields are then determined by plaque titration under permissive conditions so that the growth of mutant and wild-type virus can be quantitatively compared. Ideally, for wild-type virus, the ratio of the yield for infections done at the nonpermissive temperature relative to the permissive temperature should be one—that is, the virus should grow equally well at both temperatures. For mutant viruses, the ratio of the yield for infections done at the nonpermissive temperature relative to the permissive temperature may range from <10% to as much as 100%, even for mutants that are clearly defective in plaque formation under nonpermissive conditions. Mutants that are *tight*—or grow poorly under nonpermissive conditions—are desirable for phenotypic characterization relative to leaky mutants, because leaky mutants will logically display considerable wild-type phenotypic behavior. Special cases exist where extreme leakiness is an expected and desirable trait. Specifically, virus mutants that are wild type for replication and production of infectious virions but defective in cell-to-cell spread have a phenotype characterized by defective plaque formation, which requires spread, but 100% leakiness, which does not require spread if assayed in a high MOI one-step growth protocol.[8]

Complementation

Complementation analysis provides a general method for determining whether two different virus mutants affect the same or different genes. The quantitative test to determine complementation is a two-step procedure in which coinfections are first done to induce an interaction between two mutants, and the results of those infections are quantitatively assessed by plaque titration. The test compares the ability of two mutants to grow in mixed relative to single infections done under nonpermissive conditions. Specifically, cells are first infected with two different virus mutants at high MOI so that all cells are coinfected with both mutants, and infected cells are incubated under nonpermissive conditions where neither mutant alone can replicate, for an interval sufficient to achieve maximum virus yield. Single high MOI infections under nonpermissive conditions are performed as controls. Virus is then harvested, yields are quantified by plaque titration under both permissive and nonpermissive conditions, and a complementation index is calculated.[89] If the two mutants, A and B, are in different virus genes, then in the mixed infection done under nonpermissive conditions, mutant A can contribute wild-type B gene product and mutant B can contribute wild-type A gene product. Thus, the mutants can help or complement each other, resulting in a high yield of mutant virus in the mixed infection compared to the single infections. If the two mutants affect the same gene, then the wild-type gene product will be lacking in the mixed infection. In this case, the yield from the mixed infection will be equivalent to the yield from the single infections.

Qualitative complementation tests have also been devised for use with both bacterial and mammalian viruses.[15,20,155] These qualitative tests are much easier to perform than quantitative tests and are just as reliable. In general, the tests are designed such that bacterial lawns or eukaryotic cell monolayers are infected either singly or with two viruses under nonpermissive conditions and at relatively low MOI. The MOI must be high enough so that numerous cells are doubly infected in the mixed infection, although low enough so that most cells are uninfected and a lawn or monolayer is maintained. Complementing mutant pairs produce plaques or cleared areas under nonpermissive conditions, whereas noncomplementing mutant pairs do not. A theoretical disadvantage of the qualitative test is that it does not discriminate between complementation and recombination. If recombination does occur under nonpermissive conditions, false positives occur in the qualitative test and the number of complementation groups is overestimated.[83] Nevertheless, negative tests are still a reliable measure of noncomplementation.

Complementation analysis has been of tremendous benefit in sorting mutants in many viral systems. A notable exception is poliovirus, where complementation between temperature-sensitive mutants *in vivo* is not observed. The lack of complementation in picornaviruses may be related to the unique mechanism of viral gene expression, in which all protein products are produced from a polyprotein precursor by proteolytic cleavage. If individual temperature-sensitive mutants affect structure, synthesis, or cleavage of the polyprotein precursor, they may behave as if they all belong to a single complementation group, even though they may map to different protein end products. Additionally, functional coupling of molecular processes such as translation and RNA genome replication, and RNA replication and packaging can limit opportunities for trans-complementation of protein functions.[118,119]

As a concept, complementation impacts broadly on virology and is not limited simply to the grouping of conditionally lethal mutants into genes. For example, the growth of engineered host-range deletion mutants in essential virus genes, discussed previously, relies on complementation of the missing viral function by an engineered cell line that expresses the wild-type viral gene product. In addition, the accumulation of defective virus genomes at high multiplicity passage, also discussed earlier, results from a complementing helper function provided by wild-type virus.

Recombination and Reassortment

Recombination describes a process by which nucleic acid sequences from two genotypically different parental viruses are exchanged so that the progeny contain sequences derived from both parents. In viral systems, there exist three distinct mechanisms of recombination, dictated by the structures of the viral genomes. For DNA viruses, recombination occurs by the physical breakage and rejoining of parental DNA molecules through regions of sequence homology, in a fashion similar or identical to the same process in bacteria or higher organisms. For RNA viruses containing segmented genomes, gene exchange occurs primarily through reassortment of individual parental genome segments into progeny

viruses, although intragenic recombination has been reported for orthomyxoviruses, reoviruses, and bunyaviruses.[127,130,133,153,157] For most nonsegmented RNA viruses, recombination appears to be a much less frequent event compared with DNA viruses. Recombination has been observed in several ssRNA virus families representing both positive- and negative-sense genomes; picornaviruses, coronaviruses, togaviruses, and retroviruses display relatively efficient recombination.[4,58,71,84,85,177] Recombination in RNA viruses is thought to occur during replication via *copy choice*, namely switching templates during replication such that the newly synthesized genome contains sequence from two different parental molecules.[23,85] Historically, recombination has been used to construct genetic maps of virus mutants and to construct novel virus genotypes. Although recombination mapping has been largely replaced by physical mapping techniques, such as marker rescue, a technical knowledge of recombination mapping can contribute to an appreciation of the complexity of genetic interactions between viruses.

The methods used to determine recombination frequencies are the same regardless of genome structure or mechanism of recombination. As with complementation, the quantitative test to determine recombination frequency between two mutants, called a *two-factor cross*, is a two-step procedure, but in this case, coinfections are first done under conditions permissive for replication, and then, the fraction of recombinants relative to the total virus yield is quantitatively assessed by plaque titration. Specifically, cells are first infected with two different virus mutants at high MOI so that all cells are coinfected with both mutants, and infected cells are incubated under permissive conditions so that both mutants have maximum opportunity for interaction, for an interval sufficient to achieve maximum virus yield. Single high MOI infections under permissive conditions are performed as controls. Virus is then harvested, yields are quantified by plaque titration under both permissive and nonpermissive conditions, and a recombination frequency is calculated.[89]

Recombination mapping in DNA viruses relies on the assumption that the frequency of recombination between two genetic markers is proportional to the distance between the two markers. For several DNA viruses, observed recombination frequencies comprise a continuous range from <1% up to a theoretical maximum of 50%, allowing for construction of linear genetic maps.[51]

In viruses with segmented genomes, recombination between markers on the same segment is rare but reassortment of segments is extremely efficient; thus, recombination is effectively an all or none phenomenon, with markers on the same segment displaying no recombination, and markers on different segments displaying very high levels of recombination.[132] For these reasons, genetic exchange in segmented RNA viruses is commonly referred to as reassortment rather than recombination. Reassortment analysis for segmented viruses is useful for determining whether or not two mutations map to the same genome segment but cannot be used to determine the order of markers on a given segment. Mutations can be mapped to individual RNA segments by performing *intertypic* crosses between virus types that can be discriminated either based on differential electrophoretic mobility of each RNA segment or detection of engineered mutations unique to each parental virus.[108,132]

Marker Rescue

Marker rescue is a physical mapping technique that measures directly whether a given virus mutation maps within a specific subfragment of a virus genome. The use of marker rescue is confined to DNA viruses where homologous recombination takes place and has been of enormous value in these systems. The application of the technique varies somewhat depending on the virus system under study; however, the general principles are the same. Specifically, full-length mutant viral genomic DNA plus a wild-type DNA genomic subfragment, either a cloned DNA molecule or a PCR product, are introduced into cells under conditions permissive for recombination and for wild-type virus replication. For viruses that contain infectious DNA, such as herpesviruses,[94,154] adenoviruses,[53] and polyomaviruses,[95] the mutant genomic DNA and the wild-type genomic subfragment may be cotransfected into cells. For viruses containing noninfectious genomic DNA, such as poxviruses,[159] the mutant DNA must be introduced into cells by infection with the mutant virus, which is then followed by transfection with the wild-type DNA subfragment. In either case, the protocol allows for homologous recombination between the mutant genome and the wild-type DNA subfragment. If the wild-type DNA subfragment contains the wild-type allele for the mutation, the recombination can exchange the wild type for the mutant sequence in the mutant genome, creating wild-type virus. Conversely, if the wild-type fragment does not contain the wild-type allele for the mutation, no wild-type virus, above a background of revertants, will be created in the experiment. The presence of wild-type virus can be assayed using either a two-step or a one-step protocol. In the two-step protocol, depending on the nature of the mutation being rescued, infected and/or transfected cells are incubated under permissive conditions to facilitate recombination and replication, or nonpermissive conditions to select for wild-type recombinants, then wild-type virus yields are quantified by plaque titration under nonpermissive conditions. In the one-step protocol, the infection and/or transfection is done so that only a small fraction of the cells in a monolayer are infected, and cells are then incubated under nonpermissive conditions such that wild-type virus formed during a successful rescue will form plaques on the monolayer.[159] In short, regardless of the precise method used, conversion or *rescue* of mutant virus to wild type with a given wild-type DNA fragment means that the mutation maps within that fragment. Initial marker rescue mapping experiments may be facilitated by the use of a few large but overlapping wild-type DNA fragments, and fine mapping may be accomplished with fragments as small as a few hundred nucleotides. Marker rescue mapping has completely replaced recombination mapping as a method for mapping mutations in DNA viruses, and precise genetic maps of several DNA viruses have now been constructed.

Reverse Genetics

Prior to the advent of recombinant DNA and DNA sequencing technologies, classical genetic analysis, namely random isolation and characterization of virus mutants, was one of the few effective methods for identifying, mapping, and characterizing virus genes, and the only method for obtaining virus mutants. With the current ready availability of genomic sequences for virtually all prototypical members of each virus family and a versatile package of genetic engineering tools, the experimental landscape has changed completely. One can now conduct a genetic analysis with a reasonably complete foreknowledge of the genetic structure of the virus, focus attention on individual genes of interest, and deliberately engineer mutations in genes to study their function. Termed *reverse genetics*, this process has come to dominate the genetic analysis of viruses. Reverse genetics covers a broad range of activities ranging from engineering a single nucleotide substitution in a target gene to engineering chimeric viruses to be used as gene therapy vectors, oncolytic vectors, or vaccines. Currently, virtually every significant human viral pathogen can be engineered using reverse genetic approaches. Perhaps one of the most impressive feats in reverse genetics is the resurrection of the deadly 1918 pandemic strain of influenza using genome sequences derived from archived formalin-fixed lung autopsy materials and from frozen, unfixed lung tissues from an Alaskan influenza victim who was buried in permafrost.[163]

Reverse genetic analysis involves two distinct considerations: strategies for design of a given mutation and strategies for incorporation of mutations into virus. The principles governing these strategies highly depend on the structure of a given viral genome and the strategy of virus replication and thus vary in the extreme. However, some general principles can be identified, which are discussed next, accompanied by a few

specific examples to illustrate the general principles.

Incorporation of Mutations Into Virus

The methods used for incorporation of mutations into a virus depend on several features of the individual virus under consideration, including genome size, whether or not the nucleic acid is infectious, whether the genome is composed of DNA or RNA or replicated via reverse transcription, and whether replication is nuclear or cytoplasmic.

DNA viruses and reverse-transcribing viruses

With the exception of poxviruses, which because of their cytoplasmic site of replication must carry virion-encapsidated transcription enzymes into cells during infection, virtually all DNA virus genomes (see Table 2.1) are infectious. Likewise, double-stranded DNA (dsDNA) comprising the genomic sequences of reverse-transcribing viruses that package RNA (retroviruses) or DNA (hepadnaviruses) genomes are also infectious. Thus, in these cases, the incorporation of a mutation into the virus genome is essentially an exercise in molecular cloning, and pure mutant virus is produced by transfection of the cloned mutant genome into cultured cells. In practice, the herpesvirus genome is sufficiently large such that manipulation as a full-length genomic clone presents some difficulties, and therefore, incorporation of mutations into the viral genome is often done by cotransfecting cells with full-length genomic viral DNA along with a DNA fragment containing the desired mutant allele flanked by wild-type DNA sequences. Replication is launched from the transfected infectious wild-type genomic DNA, and homologous recombination between the cotransfected mutant DNA fragment and the wild-type genome incorporates the mutant allele into a fraction of the replicating wild-type genomes[21,180] (Fig. 2.10). A similar protocol is applied to engineering poxviruses; however, because poxvirus DNA is noninfectious, virus replication must be initiated by infection with intact virus. In its simplest form, this protocol entails infection with virus bearing the wild-type target genome followed by transfection with a DNA fragment containing the desired mutation flanked by wild-type DNA sequences[125] (Fig. 2.11; identical to the protocol for marker rescue described earlier). Similar to the herpesvirus cotransfection protocol just described, homologous recombination catalyzed by viral enzymes results in incorporation of the mutant allele into a fraction of the wild-type infecting genomes. An alternate protocol for constructing poxvirus recombinants involves first infecting cells with a replication defective, nonhomologous helper poxvirus, followed by transfection with either a cloned full-length mutant genome or a mixture of fragments comprising the desired engineered genome.[32,134,178] The helper virus provides enzymes critical for launching replication of the transfected full-length mutant viral DNA genome or for homologous recombination among transfected subgenomic fragments to assemble and launch replication of the desired virus.

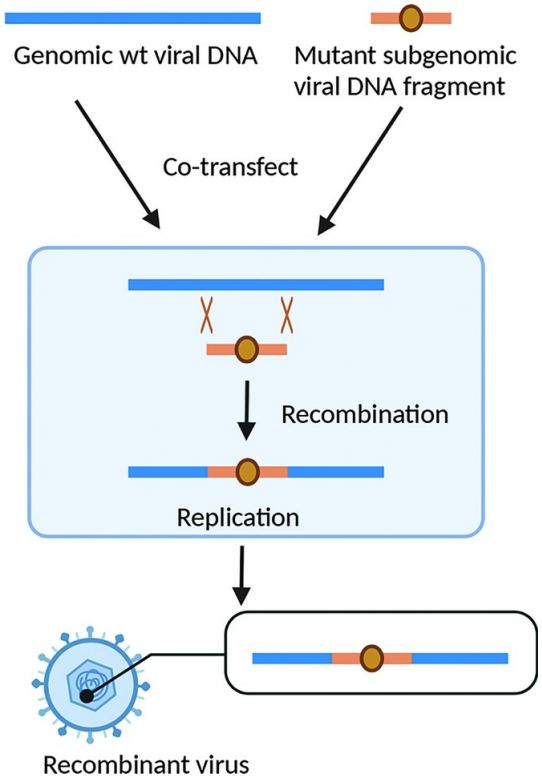

FIGURE 2.10 **Reverse genetics with large double-stranded DNA viruses containing infectious genomes.** Wild-type full-length infectious genomic viral DNA (*blue line*) is cotransfected into cells along with a subgenomic viral DNA fragment (*orange line*) containing the desired mutation (*orange circle*). Homologous recombination between the cotransfected viral DNAs takes place within the cell catalyzed by viral and/or cellular enzymes. The recombinant genome is replicated and packaged to yield recombinant virus (mixed with wild-type virus replicated from unrecombined genomes). (Created with BioRender.com.)

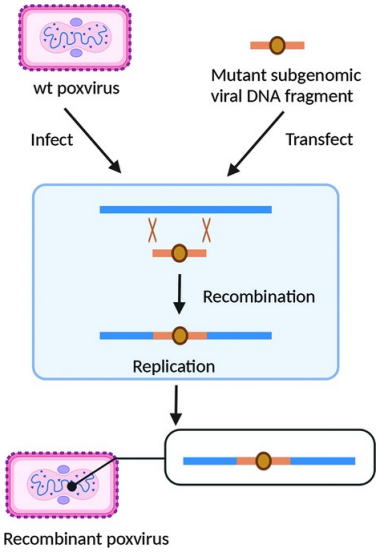

FIGURE 2.11 **Reverse genetics with large double-stranded DNA viruses containing noninfectious genomes (poxviruses).** Cells are infected with virus containing a wild-type DNA genome (*blue line*) and transfected with a subgenomic viral DNA fragment (*orange line*) containing the desired mutation (*orange circle*). Homologous recombination between the infecting viral DNA and cotransfected fragment takes place within the cell catalyzed by viral enzymes. The recombinant genome is replicated and packaged to yield recombinant virus (mixed with wild-type virus replicated from unrecombined genomes). (Created with BioRender.com.)

When mutants are engineered in cloned, infectious genomes, only the mutant allele of the target gene is present in the construction, all virus recovered from the transfection will be mutant, and no selection or screen for mutants is required. However, in situations where *in vivo* homologous recombination has been used to incorporate the cloned mutation into a wild-type genome, such as in poxviruses or herpesviruses, both mutant and wild-type viruses emerge from the mutant construction protocol, and thus, a screen or selection is required to identify the mutant of interest. For mutations in nonessential genes, this may be a relatively straightforward matter of inserting into the target gene a color marker such as β-galactosidase[180] or GFP[27] to facilitate a screen, or inserting a dominant selectable marker such as *Escherichia coli* guanine phosphoribosyltransferase[42] to facilitate a selection. For conditionally lethal phenotypes such as temperature sensitivity, although techniques exist that enrich for recombinant viruses, mutant isolation ultimately relies on a screen of individual mutants for differential growth under permissive and nonpermissive conditions.[61] The use of full-length clones of herpesvirus and vaccinia virus obviates the need for *in vivo* recombination, and thus only mutant virus will be recovered after transfection or reactivation of an engineered, mutant genome, and no mutant screen or selection is necessary.[33,111]

RNA viruses

The genomes of positive-sense ssRNA viruses (see Table 2.1) are infectious; therefore, as with most DNA viruses, the engineering of mutant viruses is largely an exercise in molecular cloning, yet formidable for large RNA viruses such as coronaviruses.[179] To recover infectious virus, complementary DNA (cDNA) clones of mutant genomes may be transfected directly into cultured cells or transcribed *in vitro* into positive-sense RNA that is then transfected into cells. Virus replication is launched by translation of the transfected RNA *in vivo*, resulting in recovery of only mutant virus. A good example of the application of this technology is the directed construction of temperature-sensitive mutants in poliovirus.[30]

Negative-sense ssRNA viruses must package in the virion a virus-coded RNA-dependent RNA polymerase so that the genome, in the form of a nucleocapsid, can be transcribed into mRNA immediately following infection. Thus, negative-sense ssRNA virus genomes are not infectious, and engineering these viruses becomes more of a challenge. Generally, the strategy consists of transfection of cells with multiple plasmids, some of which are transcribed into genome-length RNAs (encoding the desired genotype) and some of which direct expression of proteins required for genome replication, specifically a nucleocapsid protein and proteins comprising the viral RNA-dependent RNA polymerase. The expressed nucleocapsid protein encapsidates the transcribed genomic RNA, and this nucleocapsid can then be transcribed into mRNA by the expressed viral RNA polymerase, thus launching the infection and ultimately yielding pure virus of the desired genotype. Historically, the requirements for "rescue" of virus from cloned fragments are different for the segmented negative-sense ssRNA orthomyxoviruses compared to most nonsegmented negative-sense ssRNA viruses, namely rhabdoviruses, paramyxoviruses, and filoviruses, comprising most of the order *Mononegavirales*. The differences are attributable, at least in part, to the fact that the orthomyxoviruses replicate in the nucleus, whereas most of the viruses in the order *Mononegavirales* replicate in the cytoplasm. Specifically, for the orthomyxoviruses,[116] genome segments are cloned so that they are transcribed from a polymerase I promoter to yield the negative-sense genomic RNA, and the replication proteins are cloned so that they are transcribed from a polymerase II promoter to yield mRNA. Both polymerases are expressed in the cell nucleus so that after transfection of the plasmids, the viral RNAs are synthesized in the appropriate cellular compartment. The number of plasmids required for rescue can be minimized by flanking each genome segment with a polymerase I promoter at the 3′ end and a polymerase II promoter at the 5′ end so that each plasmid yields both a negative-sense genomic RNA and a positive-sense mRNA (Fig. 2.12). For most *Mononegavirales* viruses,[22] rescue is best achieved if synthesis of both the genomic RNA and the replication proteins are driven by the bacteriophage T7 RNA polymerase, which localizes efficiently to the cell cytoplasm. The T7 RNA polymerase can be supplied either by infection with a poxvirus expressing T7 RNA polymerase, by using a cell line containing a stably integrated copy of the T7 RNA polymerase gene, or by transfection of an additional plasmid designed to express the enzyme. An additional (counterintuitive) requirement for rescue of *Mononegavirales* viruses is that the plasmid encoding the genomic RNA is configured so that it is initially transcribed to yield positive-sense antigenomic RNA, which is then encapsidated with expressed nucleocapsid protein, and replicated into encapsidated negative-sense genomic RNA, which is in turn transcribed into mRNAs to launch the infection (Fig. 2.13). The use of a plasmid that expresses the negative-sense genomic RNA compromises the rescue, presumably because the negative-sense genomic RNA will hybridize in the cytoplasm with the positive-sense mRNAs for replication proteins, thus repressing their expression. Despite these generalities, examples exist of rescue of cytoplasmic *Mononegavirales* viruses using polymerase I

or polymerase II promoters.[109]

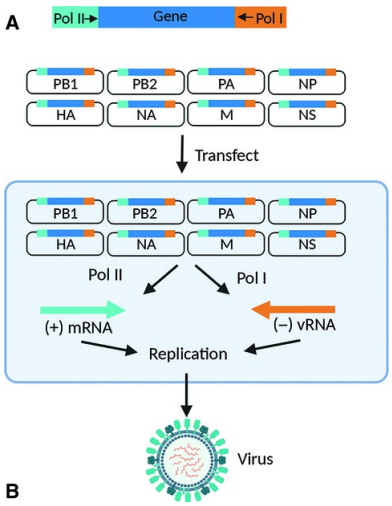

FIGURE 2.12 **Reverse genetics with orthomyxoviruses (influenza). A**. Detailed structure of cloned viral genes. Coding sequences for each viral gene (*blue*) are cloned flanked by an RNA polymerase II transcriptional promoter (*turquoise*) at the upstream (5′) end and an RNA polymerase I transcriptional promoter (*orange*) at the downstream (3′) end. **B**. Reverse genetic protocol. Cells are transfected with eight plasmids together representing the entire complement of virus genes. Transcription in the nucleus with polymerase II produces positive-sense messenger RNAs (*turquoise*) that are translated into viral proteins including the polymerase (PB1, PB2, PA) and nucleocapsid protein (NP). Transcription from the polymerase I promoter produces negative-sense genomic viral RNAs (*orange*), which are then replicated by the viral replication proteins. Further transcription, replication, and encapsidation produce virus. (Created with BioRender.com.)

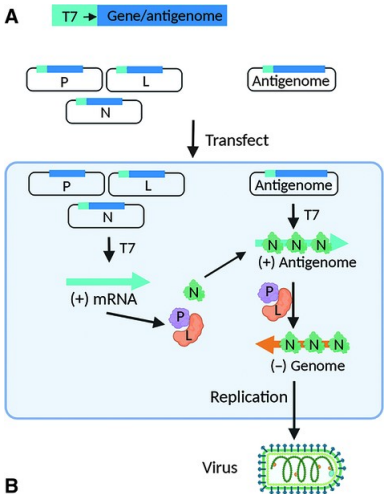

FIGURE 2.13 **Reverse genetics with viruses in the order *Mononegavirales*. A**. Detailed structure of cloned genes. Coding sequences for replication proteins or the full-length viral genome (*blue*) are cloned downstream from a bacteriophage T7 transcriptional promoter (*turquoise*). **B**. Reverse genetic protocol. Cells are transfected with four plasmids, three of which encode replication proteins (P, L, and N) and one of which contains the full-length viral genome oriented such that transcription yields a positive-sense antigenomic RNA. Transcription by T7 RNA polymerase (usually encoded by an engineered stably integrated copy in the cell nucleus) yields messenger RNA for the replication proteins (*turquoise*) plus positive-sense antigenomic viral RNA (*turquoise*). Translation of the messenger RNAs provides nucleocapsid protein (N), which encapsidates the antigenomic RNA. Antigenomic nucleocapsid is replicated by the viral polymerase (L and P) to yield negative-sense genomic viral nucleocapsid (*orange* viral RNA encapsidated with *green* N protein), which can be further transcribed and replicated to yield recombinant virus. (Created with BioRender.com.)

The segmented, double-stranded RNA (dsRNA)-containing, cytoplasmic reoviruses can be rescued using a protocol similar to that used for *Mononegavirales*.[91] Specifically, cDNAs of individual segments are cloned downstream from a bacteriophage T7 promoter, each yielding an RNA product that doubles as mRNA and the positive strand template for genomic dsDNA. Transfection of these plasmids into cells expressing T7 RNA polymerase results in synthesis of genomic segments and replication proteins, ultimately yielding pure virus of the desired genotype. T7 RNA polymerase can be supplied by any of the previously mentioned methods: cotransfection of an expression plasmid, expression from a stably integrated chromosomal gene, or infection with a poxvirus expressing the enzyme. Interestingly, this protocol did not work for all reoviruses, most notably the important human pathogen rotavirus, which, until recently, required more complex helper-mediated protocols.[162] However, an entirely plasmid-based reverse genetic system for rotavirus was developed by expressing a protein that induces cell-cell fusion and the vaccinia capping enzyme along with the viral cloned cDNAs.[82]

Mutation Design

Design of mutations for use in virology is problematic only if the gene in question is essential, necessitating isolation of a conditionally lethal mutation. For genes that are nonessential, mutation design is a simple matter of engineering a null mutation (eg, a deletion, insertion, or nonsense mutation) into the cloned gene sequence. Three basic types of engineered conditionally lethal mutations are currently in use: host-range deletion mutants, which rely on the availability of a complementing host cell; temperature-sensitive mutants constructed by clustered charge to alanine scanning; and artificially induced gene regulation. For host-range deletion mutants, the primary problem is construction of a host cell that expresses the target gene in a fashion appropriate for complementation of a null mutant in the virus. Once a cell line has been isolated, construction of the cloned mutation in the virus gene follows the same principles governing construction of a null mutation in a nonessential gene. The fundamental problem in creating temperature-sensitive mutations is that it is currently impossible to predict from primary amino acid sequence or even from three-dimensional protein structure what type of mutation will render a protein temperature sensitive. This difficulty has been partially overcome with the use of clustered charge to alanine scanning mutagenesis, in which clusters of three or more charged residues in the primary amino acid sequence of a protein are all changed to alanine.[173] In theory, charge clusters are likely to reside on the surface of the protein where they may facilitate protein-protein interactions, and neutralization of the charge by replacement with alanine may weaken such interactions without seriously disrupting the three-dimensional conformation of the protein. In practice, as much as 30% of clustered charge to alanine scanning mutants prove to be temperature sensitive, and this mutagenesis technique has been successfully used to construct temperature-sensitive mutants of both picornaviruses and poxviruses.[30,62] Lastly, conditionally lethal mutants have been constructed in poxviruses by placing essential genes under bacterial operator-repressor control in the viral genome.[136,161]

Deep Mutational Scanning

With advances in deep sequencing technologies and nucleic acid synthesis, it has become possible to query immense pools of mutated viral proteins to identify functional consequences of amino acid changes at every position in an unbiased manner.[50] This approach—*deep mutational scanning*—requires (1) a diverse library of engineered variants, (2) a system/host that links phenotype to genotype, and (3) a selection strategy. A variant library is constructed that encodes degenerate codons at every position to generate nearly all possible single amino acid substitutions. For virology applications, linking genotype to phenotype can be accomplished through engineering libraries within viral infectious clones followed by viral replication or binding assays as a phenotypic readout or by using viral protein display in yeast or mammalian cells followed by binding assays as a phenotypic readout.[115] The relative impact of each mutation is quantified by deep sequencing the variant library before selection vs after selection to determine which sequences are depleted or enriched. Deep mutational scanning has been particularly useful to examine functional consequences for mutations in viral glycoproteins, which can assist prediction of altered receptor binding, antibody binding, or membrane fusion. For example, deep mutational scanning has identified amino acid changes in SARS-CoV-2 spike protein that confer reduced antibody binding.[57]

Defective Interfering Particles

Interference refers generally to a phenomenon whereby infection by one virus results in inhibition of replication of another virus.[45] Defective interfering (DI) particle-mediated interference was first described by von Magnus,[168] who noted that serial undiluted passage of influenza virus resulted in a dramatic decrease in infectious titer while the number of particles remained constant. Essentially the same phenomenon was subsequently observed in a wide variety of RNA and DNA animal viruses, as well as in plant and bacterial viruses.[72] The mechanism of interference in each case is similar, namely virus stocks accumulate *DI particles*. DI particles are virus particles that contain genomes that are grossly altered genetically, usually by significant deletion of essential functions, but retain critical replication origins and packaging signals, allowing for amplification and packaging in coinfections with complementing wild-type *helper* virus. DI particles usually display a replication advantage relative to wild-type virus from increases in the copy number or efficiency of replication origins. DI particles actively inhibit replication of wild-type virus, presumably by competing for limiting essential replication factors. Study of DI particles has provided significant insight into viral replication, particularly structure and function of replication origins. Moreover, recent studies suggest that levels of DI genomes correlate with respiratory syncytial virus replication and disease in patient cohorts, revealing possible clinical importance.[44]

Phenotypic Mixing and Pseudotypes

If two heterologous viruses infect the same cell, then depending on the relatedness of the two viruses, the opportunity exists for packaging of either virus genome into a nucleocapsid or envelope comprised wholly or in part of structural proteins encoded by the heterologous virus. This phenomenon, termed *phenotypic mixing*, has been observed in mixed infections in a laboratory setting using both nonenveloped and enveloped viruses. Phenotypic mixing is a transient phenomenon, because infection of a cell with a single phenotypically mixed particle will result in replication and assembly only of viruses that reflect the infecting genome. Historically, phenotypic mixing experiments have contributed to understanding virus structure and assembly. There has been some speculation that phenotypic mixing may actually play a role in a natural setting, serving to maintain otherwise unfit genomes within a quasispecies over time.[175]

In mixed infections with nonenveloped viruses, phenotypic mixing has been observed between closely related adenoviruses, reoviruses, and picornaviruses.[69,133,176] Phenotypic mixing has been observed between picornaviruses as distantly related as echovirus 7 and coxsackievirus A9.[75]

In the case of enveloped viruses, phenotypic mixing consists of packaging the nucleocapsid of one virus within an envelope of a heterologous virus, and the resulting viruses are called *pseudotypes*. Pseudotype formation among enveloped viruses is relatively promiscuous, especially among retroviruses and rhabdoviruses.[181] For example, pseudotypes have been formed that contain retrovirus envelope antigens combined with genomes from rhabdoviruses, paramyxoviruses, orthomyxoviruses, herpesviruses, or coronaviruses. Conversely, pseudotypes have been formed that contain a rhabdovirus genome combined with envelope antigens from togaviruses, retroviruses, bunyaviruses, arenaviruses, paramyxoviruses, orthomyxoviruses, herpesviruses, poxviruses, or coronaviruses. Currently, the concept of phenotypic mixing and pseudotype formation is of critical utility in packaging and delivery of virus vectors, particularly because phenotypic mixing permits the tropism of a virus particle to be manipulated.[80] Pseudotyping viruses can be particularly useful in the laboratory. For example, retroviruses or vesicular stomatitis viruses pseudotyped with the SARS-CoV-2 spike attachment protein have been a useful tool because they can be used in biosafety level 2 conditions rather than the biosafety level 3 conditions required for work with bona fide SARS-CoV-2.[98,117]

REFERENCES

1. Adams MJ, Lefkowitz EJ, King AM, et al. 50 years of the International Committee on Taxonomy of Viruses: progress and prospects. Arch Virol. 2017;162(5):1441-1446.

2. Aguilera ER, Erickson AK, Jesudhasan PR, Robinson CM, Pfeiffer JK. Plaques formed by mutagenized viral populations have elevated coinfection frequencies. mBio. 2017;8(2).

3. Baltimore D. Expression of animal virus genomes. Bacteriol Rev. 1971;35(3):235-241.

4. Barr JN, Fearns R. How RNA viruses maintain their genome integrity. J Gen Virol. 2010;91(Pt 6):1373-1387.

5. Benjamin TL. Host range mutants of polyoma virus. Proc Natl Acad Sci U S A. 1970;67(1):394-399.

6. Beveridge WIB, Burnet F. The Cultivation of Viruses and Rickettsiae in the Chick Embryo. Vol 256. HM Stationery Office; 1946.

7. Bienkowska-Haba M, Luszczek W, Myers JE, et al. A new cell culture model to genetically dissect the complete human papillomavirus life cycle. PLoS Pathog. 2018;14(3):e1006846.

8. Blasco R, Moss B. Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-Dalton outer envelope protein. J Virol. 1991;65(11):5910-5920.

9. Brass AL, Dykxhoorn DM, Benita Y, et al. Identification of host proteins required for HIV infection through a functional genomic screen. Science. 2008;319(5865):921-926.

10. Bushman FD, Malani N, Fernandes J, et al. Host cell factors in HIV replication: meta-analysis of genome-wide studies. PLoS Pathog. 2009;5(5):e1000437.

11. Cai W, Schaffer PA. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J Virol. 1992;66(5):2904-2915.

12. Cello J, Paul AV, Wimmer E. Chemical synthesis of poliovirus cDNA: generation of infectious virus in the absence of natural template. Science. 2002;297(5583):1016-1018.

13. Chen YW, Huang SX, de Carvalho A, et al. A three-dimensional model of human lung development and disease from pluripotent stem cells. Nat Cell Biol. 2017;19(5):542-549.

14. Chow LT, Broker TR. In vitro experimental systems for HPV: epithelial raft cultures for investigations of viral reproduction and pathogenesis and for genetic analyses of viral proteins and regulatory sequences. Clin Dermatol. 1997;15(2):217-227.

15. Chu CT, Schaffer PA. Qualitative complementation test for temperature-sensitive mutants of herpes simplex virus. J Virol. 1975;16(5):1131-1136.

16. Coen DM, Kosz-Vnenchak M, Jacobson JG, et al. Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. Proc Natl Acad Sci U S A. 1989;86(12):4736-4740.

17. Coen DM, Schaffer PA. Two distinct loci confer resistance to acycloguanosine in herpes simplex virus type 1. Proc Natl Acad Sci U S A. 1980;77(4):2265-2269.

18. Coen DM, Weinheimer SP, McKnight SL. A genetic approach to promoter recognition during trans induction of viral gene expression. Science. 1986;234(4772):53-59.

19. Condit RC, Easterly R, Pacha RF, Fathi Z, Meis RJ. A vaccinia virus isatin-beta-thiosemicarbazone resistance mutation maps in the viral gene encoding the 132-kDa subunit of RNA polymerase. Virology. 1991;185(2):857-861.

20. Condit RC, Motyczka A. Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. Virology. 1981;113(1):224-241.

21. Conley AJ, Knipe DM, Jones PC, Roizman B. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of gamma polypeptides. J Virol. 1981;37(1):191-206.

22. Conzelmann KK. Reverse genetics of mononegavirales. Curr Top Microbiol Immunol. 2004;283:1-41.

23. Cooper P. Genetics of picornaviruses. In: Fraenkel-Conrat H, Wagner RR, eds. Comprehensive Virology. Plenum Press; 1977:133-207.

24. Cresawn SG, Prins C, Latner DR, Condit RC. Mapping and phenotypic analysis of spontaneous isatin-beta-thiosemicarbazone resistant mutants of vaccinia virus. Virology. 2007;363(2):319-332.

25. Crotty S, Maag D, Arnold JJ, et al. The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. Nat Med. 2000;6(12):1375-1379.

26. d'Herelle F. The Bacteriophage and Its Behavior. Williams & Wilkins; 1926.

27. Da Fonseca F, Moss B. Poxvirus DNA topoisomerase knockout mutant exhibits decreased infectivity associated with reduced early transcription. Proc Natl Acad Sci U S A. 2003;100(20):11291-11296.

28. Davis BD, Dulbecco R, Eisen HN, Ginsberg HS. Microbiology. J. B. Lippincott; 1990.

29. DeLuca NA, McCarthy AM, Schaffer PA. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene

encoding immediate-early regulatory protein ICP4. J Virol. 1985;56(2):558-570.

30. Diamond SE, Kirkegaard K. Clustered charged-to-alanine mutagenesis of poliovirus RNA-dependent RNA polymerase yields multiple temperature-sensitive mutants defective in RNA synthesis. J Virol. 1994;68(2):863-876.

31. Doceul V, Hollinshead M, van der Linden L, Smith GL. Repulsion of superinfecting virions: a mechanism for rapid virus spread. Science. 2010;327(5967):873-876.

32. Domi A, Moss B. Cloning the vaccinia virus genome as a bacterial artificial chromosome in Escherichia coli and recovery of infectious virus in mammalian cells. Proc Natl Acad Sci U S A. 2002;99(19):12415-12420.

33. Domi A, Moss B. Engineering of a vaccinia virus bacterial artificial chromosome in Escherichia coli by bacteriophage lambda-based recombination. Nat Methods. 2005;2(2):95-97.

34. Domingo E, Holland J, Biebricher C, et al. Quasi-species: the concept and the word. In: Gibbs A, Calisher C, Garcia-Arenal F, eds. Molecular Basis of Virus Evolution. Cambridge University Press; 1995:181-191.

35. Domingo E, Holland JJ. RNA virus mutations and fitness for survival. Annu Rev Microbiol. 1997;51:151-178.

36. Drake JW, Baltz RH. The biochemistry of mutagenesis. Annu Rev Biochem. 1976;45:11-37.

37. Drummond CG, Bolock AM, Ma C, Luke CJ, Good M, Coyne CB. Enteroviruses infect human enteroids and induce antiviral signaling in a cell lineage-specific manner. Proc Natl Acad Sci U S A. 2017;114(7):1672-1677.

38. Dubbs DR, Kit S. Isolation and properties of vaccinia mutants deficient in thymidine kinase-inducing activity. Virology. 1964;22:214-225.

39. Dulbecco R, Vogt M. Some problems of animal virology as studied by the plaque technique. Cold Spring Harb Symp Quant Biol. 1953;18:273-279.

40. Ellis EL, Delbruck M. The growth of bacteriophage. J Gen Physiol. 1939;22(3):365-384.

41. Ettayebi K, Crawford SE, Murakami K, et al. Replication of human noroviruses in stem cell-derived human enteroids. Science. 2016;353(6306):1387-1393.

42. Falkner FG, Moss B. Escherichia coli gpt gene provides dominant selection for vaccinia virus open reading frame expression vectors. J Virol. 1988;62(6):1849-1854.

43. Fathi Z, Sridhar P, Pacha RF, Condit RC. Efficient targeted insertion of an unselected marker into the vaccinia virus genome. Virology. 1986;155(1):97-105.

44. Felt SA, Sun Y, Jozwik A, et al. Detection of respiratory syncytial virus defective genomes in nasal secretions is associated with distinct clinical outcomes. Nat Microbiol. 2021;6(5):672-681.

45. Fenner F, McAuslan BR, Mims CA, et al. The Biology of Animal Viruses. Academic Press; 1974.

46. Fields BN, Knipe DM, Chanock RM, et al. Fields Virology. Raven Press; 1985.

47. Fields BN, Knipe DM, Chanock RM, et al. Fields Virology. Raven Press; 1990.

48. Fields BN, Knipe DM, Howley PM, et al. Fields Virology. Lippincott-Raven; 1996.

49. Firth C, Lipkin WI. The genomics of emerging pathogens. Annu Rev Genomics Hum Genet. 2013;14:281-300.

50. Fowler DM, Fields S. Deep mutational scanning: a new style of protein science. Nat Methods. 2014;11(8):801-807.

51. Fraenkel-Conrat H, Wagner RR. Comprehensive Virology. Plenum Press; 1977.

52. Freshney R. Culture of Animal Cells: A Manual of Basic Technique. Wiley-Liss; 2000.

53. Frost E, Williams J. Mapping temperature-sensitive and host-range mutations of adenovirus type 5 by marker rescue. Virology. 1978;91(1):39-50.

54. Garcez PP, Loiola EC, Madeiro da Costa R, et al. Zika virus impairs growth in human neurospheres and brain organoids. Science. 2016;352(6287):816-818.

55. Gerhard W, Webster RG. Antigenic drift in influenza A viruses. I. Selection and characterization of antigenic variants of A/PR/8/34 (HON1) influenza virus with monoclonal antibodies. J Exp Med. 1978;148(2):383-392.

56. Goodpasture EW, Woodruff AM, Buddingh GJ. Vaccinal infection of the chorio-allantoic membrane of the chick embryo. Am J Pathol. 1932;8(3):271-282.7.

57. Greaney AJ, Starr TN, Barnes CO, et al. Mapping mutations to the SARS-CoV-2 RBD that escape binding by different classes of antibodies. Nat Commun. 2021;12(1):4196.

58. Hahn CS, Lustig S, Strauss EG, Strauss JH. Western equine encephalitis virus is a recombinant virus. Proc Natl Acad Sci U S A. 1988;85(16):5997-6001.

59. Han J, Perez JT, Chen C, et al. Genome-wide CRISPR/Cas9 screen identifies host factors essential for influenza virus replication. Cell Rep.

2018;23(2):596-607.

60. Hartley JW, Rowe WP. Production of altered cell foci in tissue culture by defective Moloney sarcoma virus particles. Proc Natl Acad Sci U S A. 1966;55(4):780-786.

61. Hassett DE, Condit RC. Targeted construction of temperature-sensitive mutations in vaccinia virus by replacing clustered charged residues with alanine. Proc Natl Acad Sci U S A. 1994;91(10):4554-4558.

62. Hassett DE, Lewis JI, Xing X, DeLange L, Condit RC. Analysis of a temperature-sensitive vaccinia virus mutant in the viral mRNA capping enzyme isolated by clustered charge-to-alanine mutagenesis and transient dominant selection. Virology. 1997;238(2):391-409.

63. Hay AJ, Wolstenholme AJ, Skehel JJ, Smith MH. The molecular basis of the specific anti-influenza action of amantadine. EMBO J. 1985;4(11):3021-3024.

64. Hay J, Subak-Sharpe JH. Mutants of herpes simplex virus types 1 and 2 that are resistant to phosphonoacetic acid induce altered DNA polymerase activities in infected cells. J Gen Virol. 1976;31(1):145-148.

65. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961;25:585-621.

66. Heaton BE, Kennedy EM, Dumm RE, et al. A CRISPR activation screen identifies a Pan-avian influenza virus inhibitory host factor. Cell Rep. 2017;20(7):1503-1512.

67. Herfst S, Schrauwen EJ, Linster M, et al. Airborne transmission of influenza A/H5N1 virus between ferrets. Science. 2012;336(6088):1534-1541.

68. Hershey A. Spontaneous mutations in bacterial viruses. Cold Spring Harb Symp Quant Biol. 1946;11:67.

69. Holland JJ, Cords CE. Maturation of poliovirus RNA with capsid protein coded by heterologous enteroviruses. Proc Natl Acad Sci U S A. 1964;51:1082-1085.

70. Holland TC, Sandri-Goldin RM, Holland LE, Marlin SD, Levine M, Glorioso JC. Physical mapping of the mutation in an antigenic variant of herpes simplex virus type 1 by use of an immunoreactive plaque assay. J Virol. 1983;46(2):649-652.

71. Hu WS, Temin HM. Effect of gamma radiation on retroviral recombination. J Virol. 1992;66(7):4457-4463.

72. Huang AS, Baltimore D. Defective interfering animal viruses. In: Fraenkel-Conrat H, Wagner RR, eds. Comprehensive Virology. Plenum Press; 1977:73-116.

73. Hui KPY, Ching RHH, Chan SKH, et al. Tropism, replication competence, and innate immune responses of influenza virus: an analysis of human airway organoids and ex-vivo bronchus cultures. Lancet Respir Med. 2018;6(11):846-854.

74. Imai M, Watanabe T, Hatta M, et al. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature. 2012;486(7403):420-428.

75. Itoh H, Melnick JL. Double infections of single cells with ECHO 7 and coxsackie A9 viruses. J Exp Med. 1959;109(4):393-406.

76. Joklik WK, Willett HP, Amos DB, et al. Zinsser Microbiology. Appleton & Lange; 1992.

77. Jones ME, Thomas SM, Rogers A. Luria-Delbruck fluctuation experiments: design and analysis. Genetics. 1994;136(3):1209-1216.

78. Jones MK, Watanabe M, Zhu S, et al. Enteric bacteria promote human and mouse norovirus infection of B cells. Science. 2014;346(6210):755-759.

79. Jones N, Shenk T. An adenovirus type 5 early gene function regulates expression of other early viral genes. Proc Natl Acad Sci U S A. 1979;76(8):3665-3669.

80. Kafri T. Gene delivery by lentivirus vectors an overview. Methods Mol Biol. 2004;246: 367-390.

81. Kaiser AD. Mutations in a temperate bacteriophage affecting its ability to lysogenize Escherichia coli. Virology. 1957;3(1):42-61.

82. Kanai Y, Komoto S, Kawagishi T, et al. Entirely plasmid-based reverse genetics system for rotaviruses. Proc Natl Acad Sci U S A. 2017;114(9):2349-2354.

83. Kato SE, Moussatche N, D'Costa SM, et al. Marker rescue mapping of the combined Condit/Dales collection of temperature-sensitive vaccinia virus mutants. Virology. 2008;375(1):213-222.

84. Keck JG, Stohlman SA, Soe LH, Makino S, Lai MM. Multiple recombination sites at the 5'-end of murine coronavirus RNA. Virology. 1987;156(2):331-341.

85. Kirkegaard K, Baltimore D. The mechanism of RNA recombination in poliovirus. Cell. 1986;47(3):433-443.

86. Kjellen L. A variant of adenovirus type 5. Brief report. Arch Gesamte Virusforsch. 1963;13:482-488.

87. Knipe DM, Howley PM. Fields Virology. 4th ed. Lippincott Williams & Wilkins; 2001.

88. Knipe DM, Howley PM. Fields Virology. 5th ed. Lippincott Williams & Wilkins; 2007.

89. Knipe DM, Howley PM. FIelds Virology. 6th ed. Lippincott Williams & Wilkins; 2013.

90. Knipe DM, Ruyechan WT, Roizman B, Halliburton IW. Molecular genetics of herpes simplex virus: demonstration of regions of obligatory and nonobligatory identity within diploid regions of the genome by sequence replacement and insertion. Proc Natl Acad Sci U S A. 1978;75(8):3896-3900.

91. Kobayashi T, Antar AA, Boehme KW, et al. A plasmid-based reverse genetics system for animal double-stranded RNA viruses. Cell Host Microbe. 2007;1(2):147-157.

92. Konig R, Zhou Y, Elleder D, et al. Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. Cell. 2008;135(1):49-60.

93. Kuchler R. Biochemical Methods in Cell Culture and Virology. Dowden, Hutchinson & Ross; 1997.

94. Ladner JT, Wiley MR, Beitzel B, et al. A multicomponent animal virus isolated from mosquitoes. Cell Host Microbe. 2016;20(3):357-367.

95. Lai CJ, Nathans D. Mapping temperature-sensitive mutants of simian virus 40: rescue of mutants by fragments of viral DNA. Virology. 1974;60(2):466-475.

96. Lanahan MR, Maples RW, Pfeiffer JK. Tradeoffs for a viral mutant with enhanced replication speed. Proc Natl Acad Sci U S A. 2021;118(30).

97. Lancaster MA, Renner M, Martin CA, et al. Cerebral organoids model human brain development and microcephaly. Nature. 2013;501(7467):373-379.

98. Lei C, Qian K, Li T, et al. Neutralization of SARS-CoV-2 spike pseudotyped virus by recombinant ACE2-Ig. Nat Commun. 2020;11(1):2070.

99. Luria S. The frequency distribution of spontaneous bacteriophage mutants as evidence for the exponential rate of phage reproduction. Cold Spring Harb Symp Quant Biol. 1951;16:463-470.

100. Luria SE, Darnell J, Baltimore D, et al. General Virology. 3rd ed. John Wiley & Sons; 1978.

101. Luria SE, Delbruck M. Mutations of bacteria from virus sensitivity to virus resistance. Genetics. 1943;28:491-511.

102. Luria SE, Williams RC, Backus RC. Electron micrographic counts of bacteriophage particles. J Bacteriol. 1951;61:179-188.

103. Lwoff A, Home R, Tournier P. A system of viruses. Cold Spring Harb Symp Quant Biol. 1962;27:51-55.

104. Ma H, Dang Y, Wu Y, et al. A CRISPR-based screen identifies genes essential for West-Nile-Virus-induced cell death. Cell Rep. 2015;12(4):673-683.

105. Mackett M, Smith GL, Moss B. Vaccinia virus: a selectable eukaryotic cloning and expression vector. Proc Natl Acad Sci U S A. 1982;79(23):7415-7419.

106. Mahy B, Kangro, HO. Virology Methods Manual. Academic Press; 1996.

107. Margulis L. Five Kingdoms: An Illustrated Guide to the Phyla of Life on Earth. 3rd ed. W. H. Freeman; 1998.

108. Marshall N, Priyamvada L, Ende Z, Steel J, Lowen AC. Influenza virus reassortment occurs with high frequency in the absence of segment mismatch. PLoS Pathog. 2013;9(6):e1003421.

109. Martin A, Staeheli P, Schneider U. RNA polymerase II-controlled expression of antigenomic RNA enhances the rescue efficacies of two different members of the Mononegavirales independently of the site of viral genome replication. J Virol. 2006;80(12):5708-5715.

110. Matsumoto S. Rabies virus. Adv Virus Research. 1970;16:257-302.

111. Messerle M, Crnkovic I, Hammerschmidt W, Ziegler H, Koszinowski UH. Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. Proc Natl Acad Sci U S A. 1997;94(26):14759-14763.

112. Mindell DP, Villarreal LP. Don't forget about viruses. Science. 2003;302(5651):1677.

113. Murphy F. Virus taxonomy. In: Fields BN, Knipe D, Howley PM, et al., eds. Fields Virology. 3rd ed. Lippincott-Raven; 1996:15-57.

114. Murphy FA, Fauquet CM, Bishop DHL, et al. Virus Taxonomy: The Classification and Nomenclature of Viruses. The Sixth Report of the International Committee on Taxonomy of Viruses. Springer-Verlag; 1995.

115. Narayanan KK, Procko E. Deep mutational scanning of viral glycoproteins and their host receptors. Front Mol Biosci. 2021;8:636660.

116. Neumann G, Kawaoka Y. Reverse genetics systems for the generation of segmented negative-sense RNA viruses entirely from cloned cDNA. Curr Top Microbiol Immunol. 2004;283:43-60.

117. Nie J, Li Q, Wu J, et al. Establishment and validation of a pseudovirus neutralization assay for SARS-CoV-2. Emerg Microbes Infect. 2020;9(1):680-686.

118. Novak JE, Kirkegaard K. Coupling between genome translation and replication in an RNA virus. Genes Dev. 1994;8(14):1726-1737.

119. Nugent CI, Johnson KL, Sarnow P, Kirkegaard K. Functional coupling between replication and packaging of poliovirus replicon RNA. J Virol. 1999;73(1):427-435.

120. Orchard RC, Sullender ME, Dunlap BF, Balce DR, Doench JG, Virgin HW. Identification of antinorovirus genes in human cells using genome-wide CRISPR activation screening. J Virol. 2019;93(s).

121. Panicali D, Paoletti E. Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc Natl Acad Sci U S A. 1982;79(16):4927-4931.

122. Park RJ, Wang T, Koundakjian D, et al. A genome-wide CRISPR screen identifies a restricted set of HIV host dependency factors. Nat Genet. 2017;49(2):193-203.

123. Perales C, Gallego I, de Avila AI, et al. The increasing impact of lethal mutagenesis of viruses. Future Med Chem. 2019;11(13):1645-1657.

124. Perkus ME, Goebel SJ, Davis SW, et al. Vaccinia virus host range genes. Virology. 1990;179(1):276-286.

125. Perkus ME, Goebel SJ, Davis SW, Johnson GP, Norton EK, Paoletti E. Deletion of 55 open reading frames from the termini of vaccinia virus. Virology. 1991;180(1):406-410.

126. Pfeiffer JK, Kirkegaard K. A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleotide analogs via increased fidelity. Proc Natl Acad Sci U S A. 2003;100(12):7289-7294.

127. Phan TG, Okitsu S, Maneekarn N, Ushijima H. Evidence of intragenic recombination in G1 rotavirus VP7 genes. J Virol. 2007;81(18):10188-10194.

128. Pincus SE, Diamond DC, Emini EA, Wimmer E. Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C. J Virol. 1986;57(2):638-646.

129. Pinto LH, Holsinger LJ, Lamb RA. Influenza virus M2 protein has ion channel activity. Cell. 1992;69(3):517-528.

130. Plyusnin A, Kukkonen SK, Plyusnina A, Vapalahti O, Vaheri A. Transfection-mediated generation of functionally competent Tula hantavirus with recombinant S RNA segment. EMBO J. 2002;21(6):1497-1503.

131. Qian X, Nguyen HN, Song MM, et al. Brain-region-specific organoids using mini-bioreactors for modeling ZIKV exposure. Cell. 2016;165(5):1238-1254.

132. Ramig RF, Fields BN. Genetics of reoviruses. In: Joklik WK, ed. The Reoviridae. Plenum Press; 1983:197-228.

133. Ramig RF, Ward RL. Genomic segment reassortment in rotaviruses and other reoviridae. Adv Virus Res. 1991;39:163-207.

134. Rice AD, Gray SA, Li Y, Damon I, Moyer RW. An efficient method for generating poxvirus recombinants in the absence of selection. Viruses. 2011;3(3):217-232.

135. Richardson RB, Ohlson MB, Eitson JL, et al. A CRISPR screen identifies IFI6 as an ER-resident interferon effector that blocks flavivirus replication. Nat Microbiol. 2018;3(11):1214-1223.

136. Rodriguez JF, Smith GL. Inducible gene expression from vaccinia virus vectors. Virology. 1990;177(1):239-250.

137. Roizman B. Polykaryosis: results from fusion of nononucleated cells. Cold Spring Harb Symp Quant Biol. 1962;27:327-342.

138. Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc Soc Exp Biol Med. 1953;84(3):570-573.

139. Salahudeen AA, Choi SS, Rustagi A, et al. Progenitor identification and SARS-CoV-2 infection in human distal lung organoids. Nature. 2020;588(7839):670-675.

140. Sarnow P, Bernstein HD, Baltimore D. A poliovirus temperature-sensitive RNA synthesis mutant located in a noncoding region of the genome. Proc Natl Acad Sci U S A. 1986;83(3):571-575.

141. Sato T, Stange DE, Ferrante M, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology. 2011;141(5):1762-1772.

142. Sato T, Vries RG, Snippert HJ, et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature. 2009;459(7244):262-265.

143. Schnipper LE, Crumpacker CS. Resistance of herpes simplex virus to acycloguanosine: role of viral thymidine kinase and DNA polymerase loci. Proc Natl Acad Sci U S A. 1980;77(4):2270-2273.

144. Sedivy JM, Capone JP, RajBhandary UL, Sharp PA. An inducible mammalian amber suppressor: propagation of a poliovirus mutant. Cell. 1987;50(3):379-389.

145. Severson WE, Shindo N, Sosa M, et al. Development and validation of a high-throughput screen for inhibitors of SARS CoV and its application in screening of a 100,000-compound library. J Biomol Screen. 2007;12(1):33-40.

146. Shelokov A, Vogel JE, Chi L. Hemadsorption (adsorption-hemagglutination) test for viral agents in tissue culture with special reference to influenza. Proc Soc Exp Biol Med. 1958;97(4):802-809.

147. Sicard A, Pirolles E, Gallet R, et al. A multicellular way of life for a multipartite virus. Elife. 2019;8.

148. Simmonds P, Adams MJ, Benko M, et al. Consensus statement: virus taxonomy in the age of metagenomics. Nat Rev Microbiol. 2017;15(3):161-168.

149. Simpson RW, Hirst GK. Temperature-sensitive mutants of influenza A virus: isolation of mutants and preliminary observations on genetic recombination and complementation. Virology. 1968;35(1):41-49.

150. Smith EC, Blanc H, Surdel MC, Vignuzzi M, Denison MR. Coronaviruses lacking exoribonuclease activity are susceptible to lethal mutagenesis: evidence for proofreading and potential therapeutics. PLoS Pathog. 2013;9(8):e1003565.

151. Spence JR, Mayhew CN, Rankin SA, et al. Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature. 2011;470(7332):105-109.

152. Sridhar P, Condit RC. Selection for temperature-sensitive mutations in specific vaccinia virus genes: isolation and characterization of a virus mutant which encodes a phosphonoacetic acid-resistant, temperature-sensitive DNA polymerase. Virology. 1983;128(2): 444-457.

153. Steinhauer DA, Skehel JJ. Genetics of influenza viruses. Annu Rev Genet. 2002;36: 305-332.

154. Stow ND, Subak-Sharpe JH, Wilkie NM. Physical mapping of herpes simplex virus type 1 mutations by marker rescue. J Virol. 1978;28(1):182-192.

155. Studier FW. The genetics and physiology of bacteriophage T7. Virology. 1969;39(3): 562-574.

156. Summers WP, Wagner M, Summers WC. Possible peptide chain termination mutants in thymide kinase gene of a mammalian virus, herpes simplex virus. Proc Natl Acad Sci U S A. 1975;72(10):4081-4084.

157. Suzuki Y, Gojobori T, Nakagomi O. Intragenic recombinations in rotaviruses. FEBS Lett. 1998;427(2):183-187.

158. Sweet BH, Hilleman MR. The vacuolating virus SV40. Proc Soc Exp Biol Med. 1960;105:420-427.

159. Thompson CL, Condit RC. Marker rescue mapping of vaccinia virus temperature-sensitive mutants using overlapping cosmid clones representing the entire virus genome. Virology. 1986;150(1):10-20.

160. Tindle C, Fuller M, Fonseca A, et al. Adult stem cell-derived complete lung organoid models emulate lung disease in COVID-19. Elife. 2021;10.

161. Traktman P, Liu K, DeMasi J, Rollins R, Jesty S, Unger B. Elucidating the essential role of the A14 phosphoprotein in vaccinia virus morphogenesis: construction and characterization of a tetracycline-inducible recombinant. J Virol. 2000;74(8):3682-3695.

162. Trask SD, Taraporewala ZF, Boehme KW, Dermody TS, Patton JT. Dual selection mechanisms drive efficient single-gene reverse genetics for rotavirus. Proc Natl Acad Sci U S A. 2010;107(43):18652-18657.

163. Tumpey TM, Basler CF, Aguilar PV, et al. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science. 2005;310(5745):77-80.

164. Van-Regenmortel M. Virus species, a much overlooked but essential concept in virus classification. Intervirology. 1990;31:241-254.

165. Van-Regenmortel MH, Fauquet C, Bishop CM, et al. Virus Taxonomy: The Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press; 2000.

166. Villarreal LP. Are viruses alive? Sci Am. 2004;291(6):100-105.

167. Vogt P. Focus assay of Rous sarcoma virus. In: Habel K, Salzman N, eds. Fundamental Techniques in Virology. Academic Press; 1969:198-211.

168. Von Magnus P. Incomplete forms of influenza virus. Adv Virus Res. 1954;2:59-79.

169. Walker PJ, Siddell SG, Lefkowitz EJ, et al. Changes to virus taxonomy and to the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses (2021). Arch Virol. 2021;166(9):2633-2648.

170. Wang Y, Pfeiffer JK. Emergence of a large-plaque variant in mice infected with coxsackievirus B3. mBio. 2016;7(2):e00119.

171. Watson DH, Russell WC, Wildy P. Electron microscopic particle counts on herpes virus using the phosphotungstate negative staining technique. Virology. 1963;19:250-260.

172. Weisblum Y, Schmidt F, Zhang F, et al. Escape from neutralizing antibodies by SARS-CoV-2 spike protein variants. Elife. 2020;9.

173. Wertman KF, Drubin DG, Botstein D. Systematic mutational analysis of the yeast ACT1 gene. Genetics. 1992;132(2):337-350.

174. Wildy P. Classification and nomenclature of viruses: first report of the International Committee on Taxonomy of Viruses. Monogr Virol. 1971;5:1-181.

175. Wilke CO, Novella IS. Phenotypic mixing and hiding may contribute to memory in viral quasispecies. BMC Microbiol. 2003;3:11.

176. Williams J, Young H, Austin P. Complementation of human adenovirus type 5 ts mutants by human adenovirus type 12. J Virol. 1975;15(3):675-678.

177. Worobey M, Holmes EC. Evolutionary aspects of recombination in RNA viruses. J Gen Virol. 1999;80(Pt 10):2535-2543.

178. Yao XD, Evans DH. High-frequency genetic recombination and reactivation of orthopoxviruses from DNA fragments transfected into leporipoxvirus-infected cells. J Virol. 2003;77(13):7281-7290.

179. Yount B, Denison MR, Weiss SR, Baric RS. Systematic assembly of a full-length infectious cDNA of mouse hepatitis virus strain A59. J Virol. 2002;76(21):11065-11078.

180. Yu D, Sheaffer AK, Tenney DJ, Weller SK. Characterization of ICP6::lacZ insertion mutants of the UL15 gene of herpes simplex virus type 1 reveals the translation of two proteins. J Virol. 1997;71(4):2656-2665.

181. Zavada J. The pseudotypic paradox. J Gen Virol. 1982;63 (Pt 1):15-24.

182. Zhang R, Kim AS, Fox JM, et al. Mxra8 is a receptor for multiple arthritogenic alphaviruses. Nature. 2018;557(7706):570-574.

183. Zhou H, Xu M, Huang Q, et al. Genome-scale RNAi screen for host factors required for HIV replication. Cell Host Microbe. 2008;4(5):495-504.

184. Zwillinger D. CRC Standard Mathematical Tables and Formulae. 30th ed. CRC Press; 1996.

CHAPTER 3

Principles of Virus Structure

Stephen C. Harrison

Introduction

How virus structures are studied

Virus symmetry

Structure and assembly of closed shells

Quasi-equivalent icosahedral arrangements

Other types of icosahedral surface organization

Frameworks and scaffolds

Mass production and specificity

Elongated shells

Multishell particles

Rearrangements in surface lattices

Two recurring globular-domain structures in icosahedral capsid proteins

Genome packaging

Positive-strand RNA genomes

dsDNA genomes

dsRNA genomes

Negative-strand RNA genomes

Viral membranes

Postassembly steps

Structural biology of virus entry

Receptors and coreceptors

Membrane fusion

Penetration by nonenveloped viruses

Perspective

Introduction

Virus particles are self-propagating carriers of genetic material. They are, in effect, extracellular organelles that pass nucleic acid–encoded information from one cell to another. They contain most of the molecular machinery necessary for efficient and specific packaging of viral genomes, escape from an infected cell, survival of transfer to a new host cell, attachment, penetration, and initiation of a new replication cycle. This molecular machinery generally works by co-opting more elaborate elements of a host cell's apparatus for carrying out related processes.

Various organizational modes have evolved to perform the functions just outlined. The most critical distinction, from a structural perspective, is between enveloped viruses—those with lipid-bilayer membranes—and nonenveloped viruses—those without such membranes. Both categories include well-known human pathogens. Examples of the former include influenza virus, HIV-1, and SARS-CoV-2. Examples of the latter include poliovirus, adenoviruses, and papillomaviruses.

The lipid bilayer of enveloped viruses is an effective protective barrier for the viral genome. Most enveloped virus particles acquire their membrane during exit from the cytosol. This step generally proceeds by budding of the assembling particle through a cellular membrane, which can be either the plasma membrane (eg, for influenza virus) or the membrane of an internal organelle (eg, the endoplasmic reticulum for dengue virus). Entry includes the reverse of budding—fusion of the viral enveloped with a cell membrane, either at the cell surface or in an internal, endocytic compartment, such as an endosome.

The tightly organized protein shell ("capsid") of a nonenveloped virus is, like the bilayer of an enveloped virus, a shield that protects the viral genome, as well the agent of viral attachment. The virus particles ("virions") assemble in either the cytoplasm or the nucleus, and in most cases

cell lysis is probably the principal route of release. Some nonenveloped viruses can be released within membrane "packages"[119,414]; the incidence of this mode of release may be more extensive than currently recognized.[205] Nonenveloped virus particles must also breach a membrane to translocate into the cytosol, and uptake by some mode of endocytosis appears to ensure that any membrane lesion during the penetration step does not allow damaging molecular leakage in either direction or major dissipation of the cell's resting potential.

Most of this chapter will illustrate with specific examples the notions just outlined, emphasizing not only the structural principles but also the importance of molecular structure for understanding relevant mechanisms in the infectious cycle. The examples, chosen to exemplify specific structure-function relationships, are by no means exhaustive, and the chapters on individual virus families contain many more relevant details, including structures of viral enzymes—proteases and polymerases—not covered here.

How Virus Structures Are Studied

Electron microscopy has been, since the 1940s, the most direct way to determine the general morphology of virus particles. Traditional thin-sectioning methods continue to be useful for examining infected cells. The thickness of a section and the coarseness of staining methods limit resolution to about 50-75 Å, even in the best cases. ("Resolution" means the approximate minimum size of a substructure that can be detected or separated in an image from its neighbor. Recall that a carbon-carbon bond length is about 1.5 Å; the diameter of an α-helix, 10 Å; and the diameter of a DNA double helix, 20 Å.) Negative staining, with uranyl acetate, potassium phosphotungstate, or related, electron-dense compounds, gives more detailed images of isolated and purified virus particles. Viruses embedded in negative stain are often relatively well preserved.[86] The electron beam destroys the particle itself very rapidly, but it leaves the dense "cast" of stain undamaged for much longer. If the particle is fully covered by the negative stain, the image contains contrast from both the upper and the lower surface of the particle, and interpretation of finer aspects of the image requires modeling or computational analysis.[88] The three-dimensional image reconstruction methods now used routinely for cryogenic electron microscopy (cryo-EM—see next paragraph) were first implemented to interpret "two-sided" images of negatively stained virus particles.[87,101,210]

Methods for preserving viruses and other macromolecular assemblies by rapid freezing to cryogenic temperatures have permitted visualization of electron-scattering contrast from the structures in the particle itself and not just from the envelope created by the surrounding cast of negative stain.[4,15,318] Moreover, regular images can be selected from a heterogeneous field, allowing study of unstable or relatively impure preparations. Rapid advances in instrumentation and in computational methods have enabled cryo-EM three-dimensional density maps at resolutions comparable to those achieved by x-ray crystallography.[143,167,214] An example from even before the most recent innovations is illustrated in Figure 3.1.[415] Such "image reconstructions" are obtained by combining information from thousands of different images of individual particles. The combination is possible, because the particles of many viruses (eg, the papillomavirus particles in Fig. 3.1) are all the same. When such uniformity is not present, for example, as in the case of a complete herpesvirus particle rather than an isolated nucleocapsid, then information from different particles cannot be combined directly. A tomographic tilt series of images from a single particle (analogous to a CAT scan in medical radiography) can be recorded instead (cryogenic electron tomography or "cryo-ET").[225] The resulting three-dimensional image is of lower resolution, as electron damage limits the information that can be obtained from an individual particle, even when the data are taken at liquid-nitrogen or liquid-helium temperatures. Tomographic reconstructions can nonetheless be very useful, as illustrated in Figure 3.2A. Averaging the images of defined substructures within a tomogram or among many tomograms ("subtomogram averaging") can yield a more detailed representation, in favorable cases at a resolution that allows fitting an atomic model (Fig. 3.3).[399]

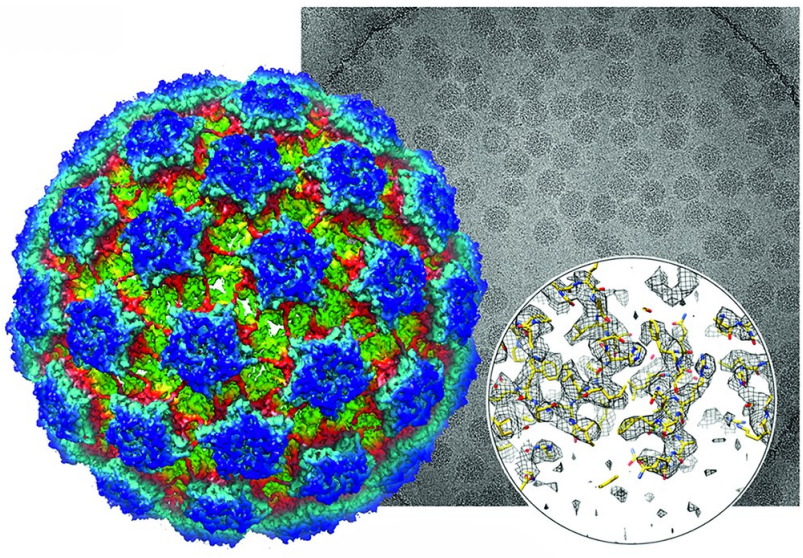

FIGURE 3.1 **Cryogenic electron microscopy (cryo-EM) of bovine papillomavirus (BPV)**. In the foreground is a color rendering of the three-dimensional image reconstruction, based on the kinds of micrographs shown in the background picture. The *circular inset at lower right* illustrates that this reconstruction provides information that extends to a nearly atomic level of detail ("resolution"); it shows a small part of the density map that resulted from the image analysis and the fit to that map of parts of the L1 polypeptide chain. (See refs.[143,415])

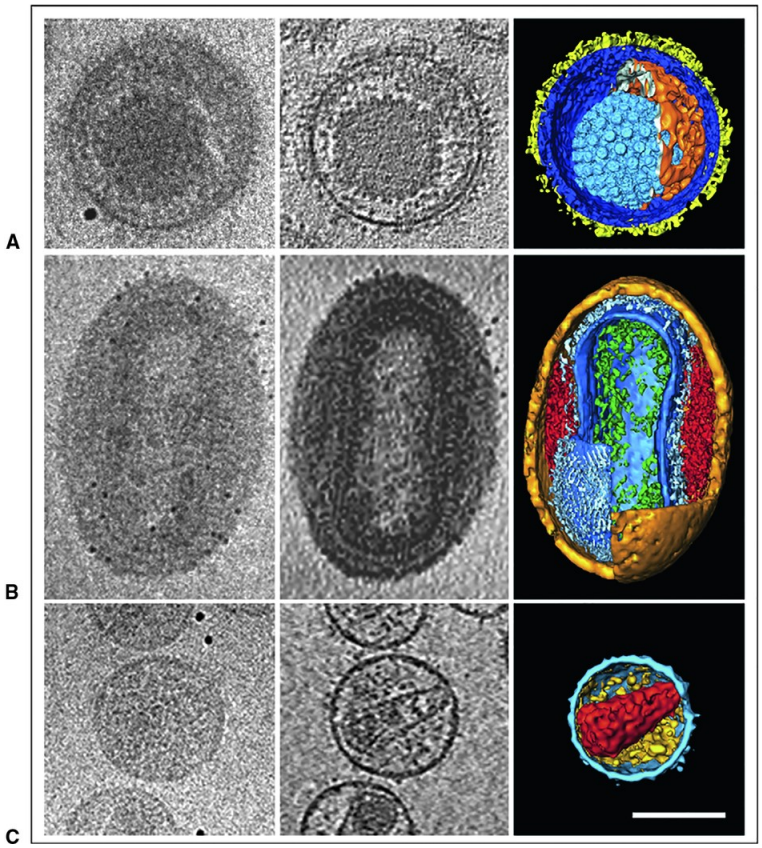

FIGURE 3.2 **Cryogenic electron tomography (cryo-ET) of herpes simplex virus type 1 (*top row*),[147] vaccinia intracellular mature virion (*middle row*),[90] and HIV-1 (*bottom row*).[37]** Images in the *left-hand column* are single, projected images; those in the *middle column*, slices through the reconstructed tomogram; those on the *right*, cut-away surface renderings of the three-dimensional tomographic reconstructions. (Reprinted from Grunewald K, Cyrklaff M. Structure of complex viruses and virus-infected cells by electron cryo tomography. *Curr Opin Microbiol*. 2006;9(4):437-442. Copyright © 2006 Elsevier, with permission. Ref.[146a])

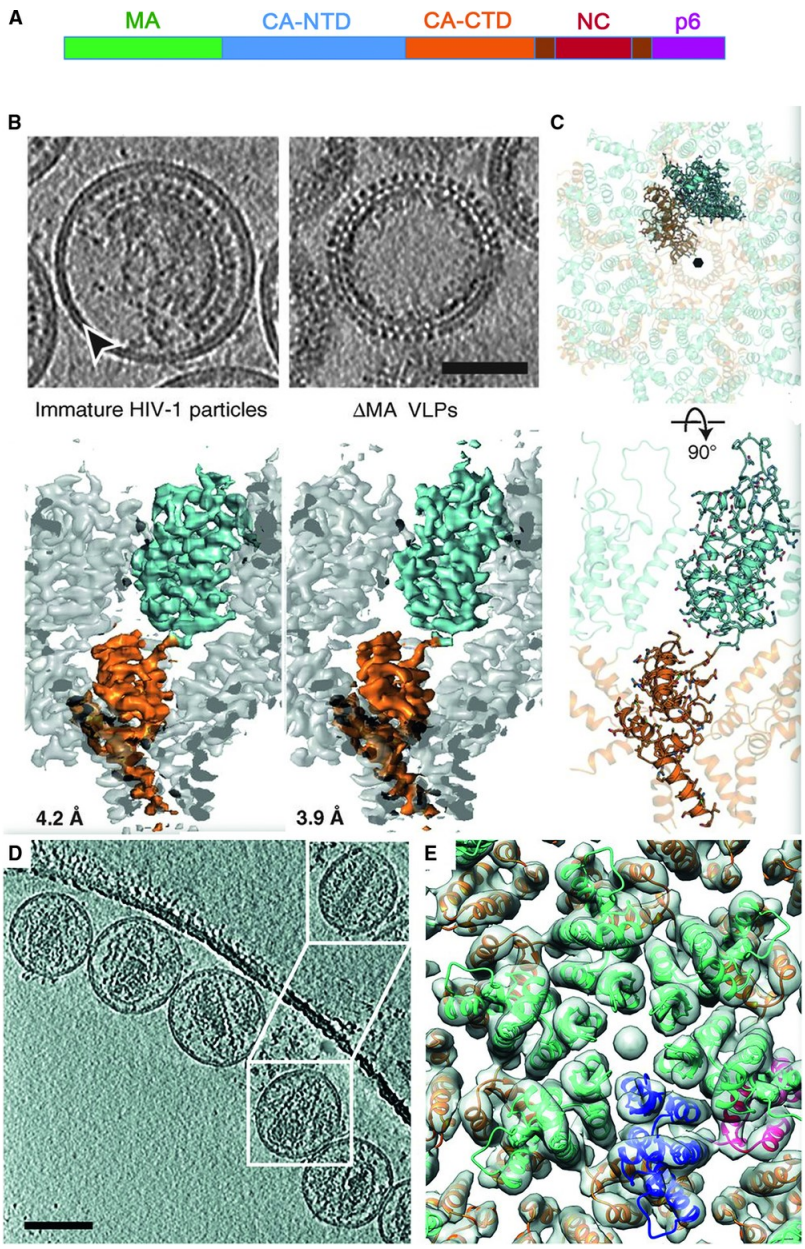

FIGURE 3.3 **Cryo-ET and subtomogram averaging of immature and mature HIV-1 particles**. **A**. Diagram of HIV-1 Gag domains, for reference. MA, matrix; CA, capsid; NC, nucleocapsid. See also Fig. 3.24. **B**. Computational slices through cryotomograms of immature HIV-1 particles (*arrowhead* points to membrane bilayer) and viruslike particles (VLPs) lacking the MA domain of Gag (ΔMA). Without MA, the particles do not bud and do not acquire a membrane. The CA domain in the array of Gag subunits (either attached to membrane or, in the ΔMA VLP, projecting at the surface) is particularly prominent. Scale bar: 50 nm. Below each tomogram is a surface rendering of the CA lattice, determined by averaging many local regions from multiple particles ("subtomogram averaging"); one CA subunit is in color, with NTD and CTD as in **(A)**. The resolution is good enough to fit an atomic model, as shown in **(C)**, viewed face-on to the lattice (*top*) and normal to it (*bottom*, same view as in **(A)**). (**B-C** adapted from Schur FK, Obr M, Hagen WJ, et al. An atomic model of HIV-1 capsid-SP1 reveals structures regulating assembly and maturation. *Science*. 2016;353(6298):506-508. Reprinted with permission from AAAS. Ref.[348]) **D**. Slice through a tomogram of mature HIV-1 particles. Cleavage of the Gag precursor has separated CA from MA, leading to rearrangement of CA into a fullerene cone (see Figs. 3.17B and 3.24). Scale bar: 100 nm. **E**. Subtomogram averaging of the CA hexamers yields a subnanometer resolution map, into which the backbone structure of CA has been docked. (**D-E** adapted from Mattei S, Glass B, Hagen WJ, et al. The structure and flexibility of conical HIV-1 capsids determined within intact virions. *Science*. 2016;354(6318):1434-1437. Reprinted with permission from AAAS. Ref.[253])

A limitation of current cryo-EM methods is the molecular size of the smallest particle for which a high-resolution structure can be obtained.[166] That limit is roughly 100 kDa, although special techniques allow recording of high-resolution data from even smaller proteins or protein complexes. Most trimeric spike proteins of enveloped viruses are substantially larger than 100 kDa, and most viral polymerases are also amenable to structure determination by cryo-EM.[135,172,231,292] The very large number of cryo-EM structures of SARS-CoV-2 spike protein bound with Fabs of various antibodies illustrates the power of this approach.[191,430] When multiple states of a protein are present in the same sample, each one uniform enough to yield a good structure, image analysis methods permit computational classification, thus yielding structures of each state. X-ray crystallography continues to be the method of choice for obtaining structures of smaller viral enzymes, especially if high-resolution details of inhibitor complexes are a goal; it is likewise an appropriate method for structures of complexes of domains of viral glycoproteins with antibody Fabs.

Virus Symmetry

The coding capacity of a viral genome and the size of the shell needed to surround it together require that a virus particle contain many copies of one or a few protein subunits. In 1956, Crick and Watson realized that small viruses seen by electron microscopy were either rods or spheres.[85] The regularity of these particles led them to propose that they would be symmetrical assemblies, with either helical symmetry of some kind (for the rods, such as tobacco mosaic virus—TMV) or one of the three kinds of cubic symmetry (for the spherical particles, such as turnip yellow mosaic virus—TYMV). In a paper that followed theirs, Caspar showed by analysis of x-ray diffraction patterns that tomato bushy stunt virus (TBSV) has icosahedral symmetry, which is the most economical of the cubic symmetries.[57] An icosahedrally symmetric shell contains a greater number of symmetry-related subunits[54] than does a shell of tetrahedral[143] or octahedral[191] symmetry—the other two closed-shell possibilities. Hence, to contain a genome of a particular size, it dedicates fewer nucleotides of its genome to encoding the package in which they arrive than does either of the other two, leaving more room to encode other proteins, such as a viral polymerase.

Symmetry of an assembly means that certain "operations," such as rotation, relate identical configurations (Fig. 3.4). Proteins are chiral, so operations such as reflection in a plane are not possible. The symmetry operations permitted for chiral, closed-shell structures are, therefore, just rotations about axes running through the center of the particle. Icosahedrally symmetric structures have 12 fivefold axes (ie, axes about which a rotation of 72° brings the structure into self-coincidence), 20 threefold axes (120° rotation), and 30 twofold axes (180° rotation). These operations relate any one subunit to all 59 others (Fig. 3.4). The helical symmetry operations of rodlike structures have both rotational and translational components (Fig. 3.5) and can generate a helical rod of arbitrary length.

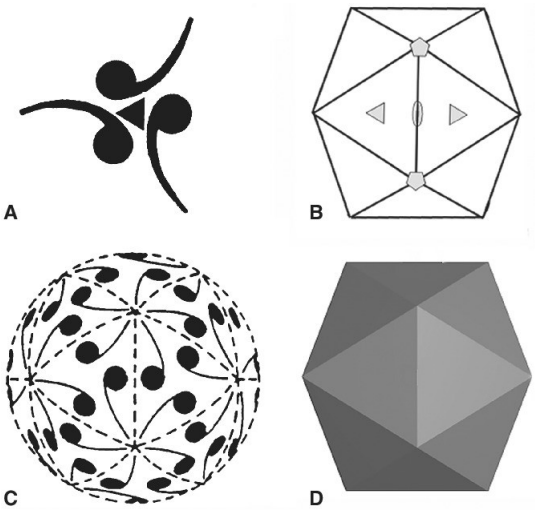

FIGURE 3.4 **Icosahedral symmetry**. **A**. Threefold symmetry: the three commas are related to each other by 120° rotations about the central axis, marked by a small triangle. **B**. Outline of an icosahedron, showing positions of some of the symmetry axis (imagined to extend from center of the icosahedral to the point on the surface marked by the symbol): fivefold, threefold, and twofold axes are marked by pentagons, triangles, and an oval, respectively. **C**. A n icosahedrally symmetry arrangement of commas on the surface of a sphere. For locations of symmetry axes, compare with panel **(B)**. **D**. Shaded surface view of an icosahedron.

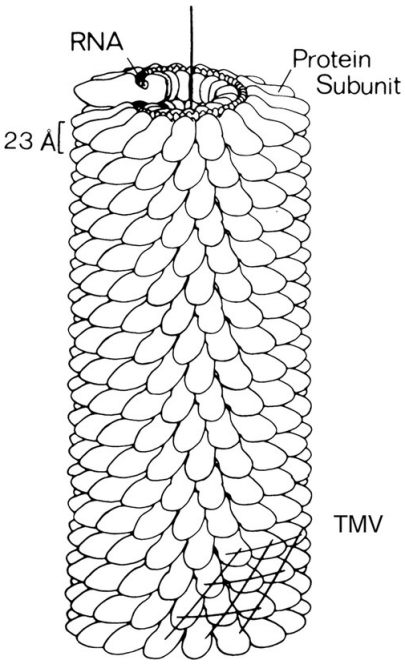

FIGURE 3.5 **Helical symmetry**. Diagram of the tobacco mosaic virus (TMV) particle. The elongated "loaves," with a groove for the RNA, represent the protein subunits. Three RNA nucleotides fit into the groove on each subunit. There are 16 1/3 subunits per turn of the

right-handed helix (ie, 49 subunits in three turns), with a rise of 23 Å as indicated. At the *lower right*, the surface lattice is drawn onto the outer particle. (Adapted from a drawing by Caspar DLD. Assembly and stability of the tobacco mosaic virus particle. *Adv Protein Chem*. 1963;18:37-121. Copyright © 1963 Elsevier, with permission. Ref.[56])

Symmetrical structures have the important property that all subunits interact identically with their neighbors and that stability of these interactions under intracellular conditions can drive self-assembly within an infected cell. In the years following the 1956 papers, it had become clear that many small, spherical viruses, such as TBSV and TYMV, contained more than 60 subunits, and Caspar and Klug proposed in 1962 that icosahedrally symmetric shells composed of certain multiples of 60 subunits would have the property that they could have nearly identical interactions among the subunits, requiring only modest flexibility in the contacts or in the subunits themselves, as shown in Figure 3.6. They called these nearly identical interactions "quasi-equivalent" and the multiples of 60 compatible with quasi-equivalent contacts, the "triangulation number" ("T-number") of the corresponding surface lattice (see Fig. 3.6 and section on quasi-equivalence, below).

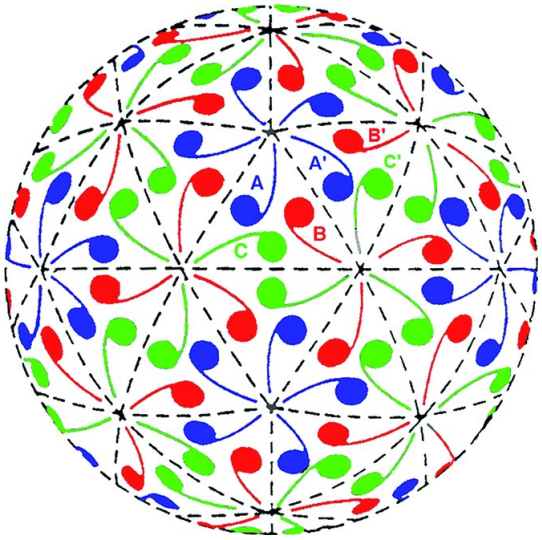

FIGURE 3.6 **Quasi-equivalent arrangement of 180 commas, in a T = 3 icosahedral surface lattice on a sphere**. Compare Fig. 3.4C, a T = 1 arrangement of 60 commas with icosahedral axes oriented similarly. The three quasi-equivalent positions within a single icosahedral asymmetric unit are shown in *blue*, *red*, and *green* and labeled *A*, *B*, and *C*, respectively, in two of the asymmetric units.

Structure AND ASSEMBLY of Closed Shells

With a typical, compact protein domain of 250-300 amino acid residues, close to the upper limit for most single protein domains, what sort of icosahedrally symmetric container can we construct? Suppose that the protein is so shaped that 60 copies fit together into a 30-Å thick shell with no significant gaps. Then the cavity within that shell will have a radius of about 80 Å, which can contain a 3-4 kb piece of single-strand DNA or RNA, tightly condensed. Some very simple virus particles conform to this description. The parvoviruses (see Chapter 6, volume 2) contain a 5.3 kb ssDNA genome, and their shells have 60 copies of a protein of ~550 residues[260] (Fig. 3.7). The capsid protein, therefore, uses up about one-third of the genome. ("Capsid," from the Latin *capsa*, box, designates the protein shell that directly packages DNA or RNA; "nucleocapsid" refers to the shell plus its nucleic-acid contents.) Likewise, the satellite of tobacco necrosis virus (STNV) contains 60 copies of a 195 residue subunit and a 1120 bp ssRNA genome, of which over half is used for the coat protein.[234] As the name implies, however, STNV is actually a defective virus, and it requires tobacco necrosis virus coinfection to propagate.

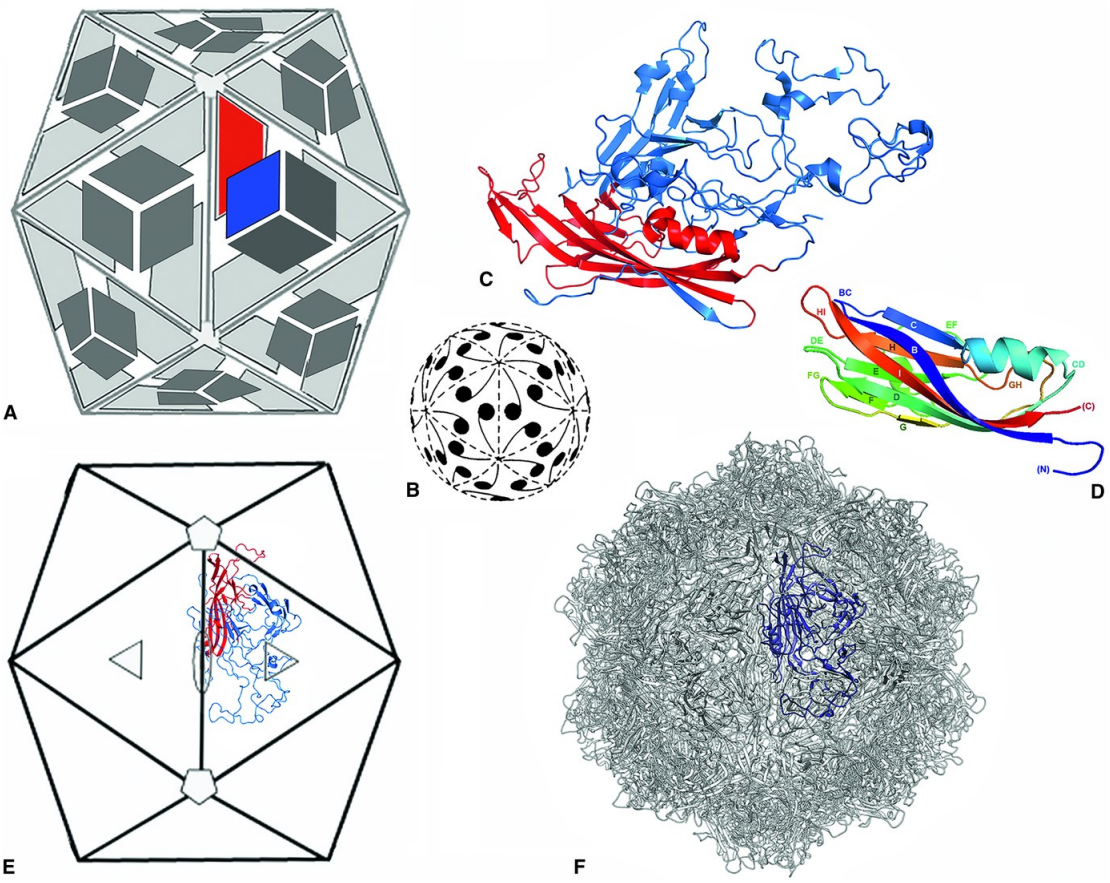

FIGURE 3.7 **Canine parvovirus (CPV): a simple, icosahedrally symmetric virion**. **A**. Icosahedron, viewed along a twofold axis, with diagrammatic representations of a protein subunit with a core domain (colored *red* on one of the subunits) and a projecting region (*blue*). Compare the subunits with the representation of commas in **(B)**, repeated from Figure 3.4C. **C**. Ribbon diagram of the CPV protein subunit; the core domain (*red*) is a β-jelly roll, from which emanate several loops that cluster to form a complex projecting region (*blue*). The simplified representation of the β-jelly roll in **(D)** is in rainbow coloring, from *blue* at its N-terminus to *red* at its C-terminus. The eight strands are lettered B-I; the loops have the letters of the strands they connect. The projecting region of the CPV subunit comprises loops BC, EF, and GH, and a short strand A, leading into the main β-jelly roll, is evident in the foreground. **E.** Icosahedron, as in **(A)**, but with a ribbon representation of one subunit; symbols for symmetry axes as in Figure 3.3B. **F**. Ribbon representation of all 60 subunits, with the subunit from **(E)** in *blue* and all others in *gray*.

More complex viruses have evolved ways to make larger, icosahedrally symmetric shells without expending unnecessary genetic resources. The simplest, but least economical, is just to use several different subunits, each of "garden-variety" size, to make up one icosahedral asymmetric unit. The picornaviruses (polioviruses, rhinoviruses, etc.) have 60 copies of three distinct proteins, VP1, VP2, and VP3, each of between 230 and 300 amino acid residues, as well as 60 copies of a small internal peptide, VP4 (see Fig. 3.8 and Chapter 2, volume 1). The shell has a cavity about 95 Å in diameter, which holds an RNA genome of 7.5-8 kb. The picornaviruses thus expend about one-third of their genome to encode the structural proteins of the virion. (The term "virion" means "virus particle," generally implying the mature, infectious structure.) We note here two other important features of picornavirus molecular architecture. First, the folded structures of VP1, VP2, and VP3 all have the same core structure—a domain known as a "jelly-roll β-barrel"[174,337] (Figs. 3.7 and 3.8). The single subunits of the parvoviruses and of STNV have the same basic fold. It is a module particularly well suited to the formation of closed, spherical shells, because of its blocklike, trapezoidal outline, but its prevalence among viral subunits may be evidence of a deeper evolutionary relationship. A second noteworthy feature of picornavirus design is that armlike extensions of the subunits tie together the assembled particle (Fig. 3.8). The importance of scaffoldlike intertwining of subunit arms was first discovered in the simple plant viruses.[163] Folding of part of the subunit and assembly of the shell are concerted processes.

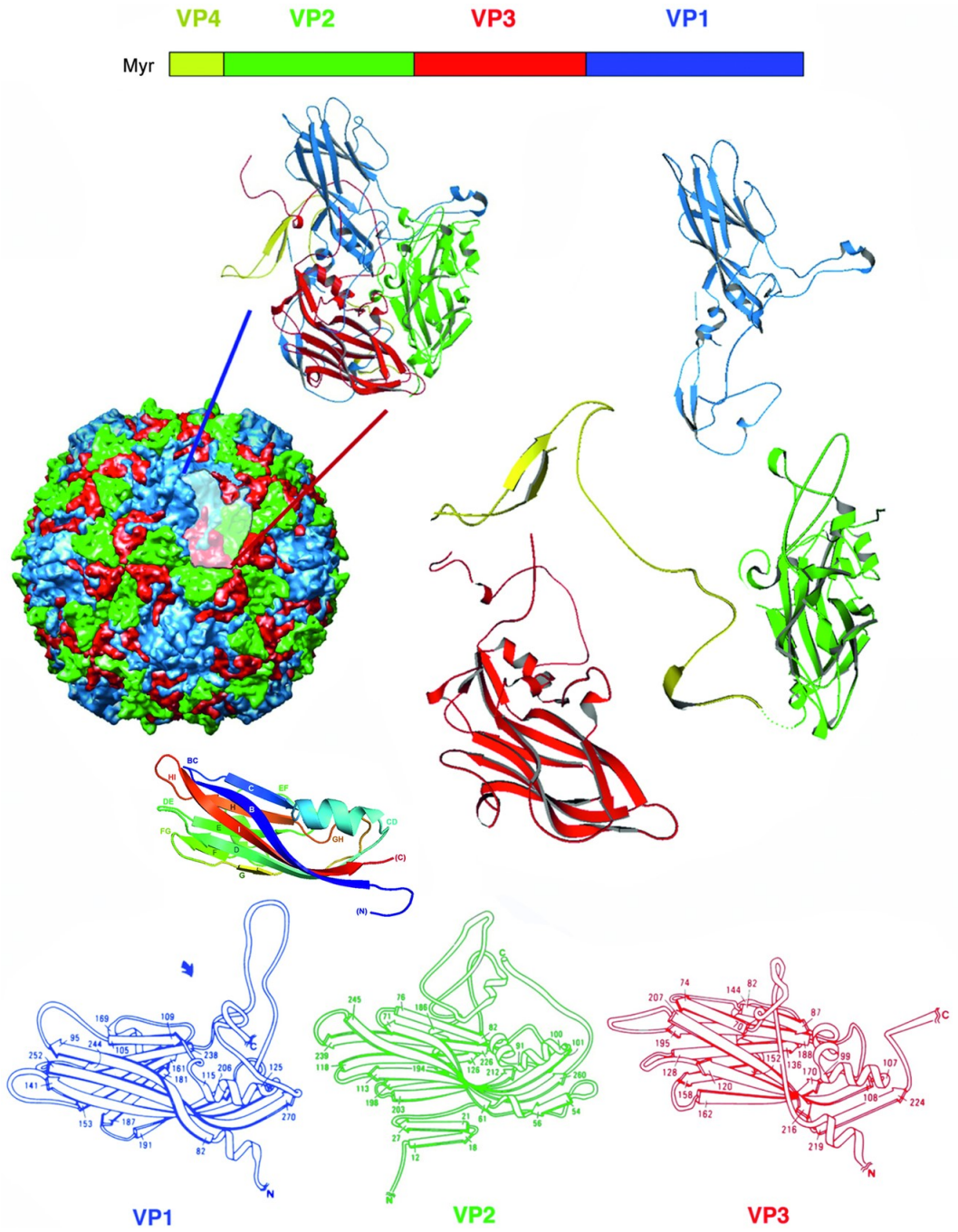

FIGURE 3.8 **Poliovirus**. *Top*: The order of structural proteins in the polyprotein encoded by the viral RNA. These domains are at the N-terminal end of the polyprotein, which is modified by myristoylation (Myr). The viral protease that cleaves between VP0 (=VP4 + VP2) and VP3 and between VP3 and VP1 is encoded by a region 3′ to the region that encodes the structural proteins; the VP4-VP2 cleavage is autolytic and occurs only after assembly of the virion precursor. *Middle*: Surface representation of the virus particle, with colors as in the bar diagram at the top. Two successively "exploded" views of an icosahedral asymmetric unit ("protomer") are shown next to the surface rendering. VP1, VP2, and VP3 each have a central β-jelly roll, with variable interstrand loops and variable N- and C-terminal extensions. The rainbow-colored β-jelly roll below the surface view is repeated from Figure 3.5D. *Bottom*: Side-by-side views of the β-jelly roll domains of VP1, VP2, and VP3, to illustrate their congruence.

Quasi-equivalent Icosahedral Arrangements

An economical way to build shells from more than 60 average-sized, identical subunits[59] is illustrated by the diagram of 180 commas in Figure 3.6. The commas have similar interactions (head to head in pairs; neck to neck in rings of three; tail to tail in rings of five or six), but they fall into three sets, designated A, B, and C. If the commas are taken to represent proteins, then the conformational differences between positions A and B, for example, involve the differences between rings of five and rings of six, for contacts involving the parts of the proteins symbolized by the tails. Protein subunits have the capacity for conformational switching needed to accommodate in this way to somewhat different packing environments, without sacrificing specificity. The alternative conformational states of a subunit then allow it to conserve many features of its interfaces with neighboring subunits ("quasi"-equivalence, as opposed to "strict" equivalence of interactions related to each other by the overall symmetry of the structure).[59]

Many plus-sense, ssRNA plant and animal viruses conform to this description of quasi-equivalent arrangements. In TBSV[163] and Norwalk and other caliciviruses,[313] there are 180 genetically and chemically identical subunits in the capsid. The subunits are actually larger than those of the picornaviruses, but most of the extra size comes from a second, projecting domain that serves functions other than the construction of a closed shell. The size of the "shell domain" ("S-domain") in both cases is about 200 residues, and the folded structure of the domain is a jelly-roll β-barrel. The TBSV diagram in Figure 3.9 illustrates important feature of the packing of these 180 S-domains. The contents of an icosahedral asymmetric unit can be described as three chemically identical subunits, with somewhat different conformations. These conformers are denoted A, B, and C, echoing the designation of commas in Figure 3.6. The principal differences among the conformers are an ordered or disordered conformation for part of the N-terminal arm and the hinge angle between the S-domain and the projecting, "P-domain." The A and B conformations are nearly identical, with disordered arms and similar hinge angles. The C conformation has an ordered arm and a different hinge angle from A and B. The ordered arms extend along the base of the S-domain and intertwine with two others around the icosahedral threefold axis. Thus, the whole collection of 60 C-subunit arms forms a coherent inner scaffold (Fig. 3.9).

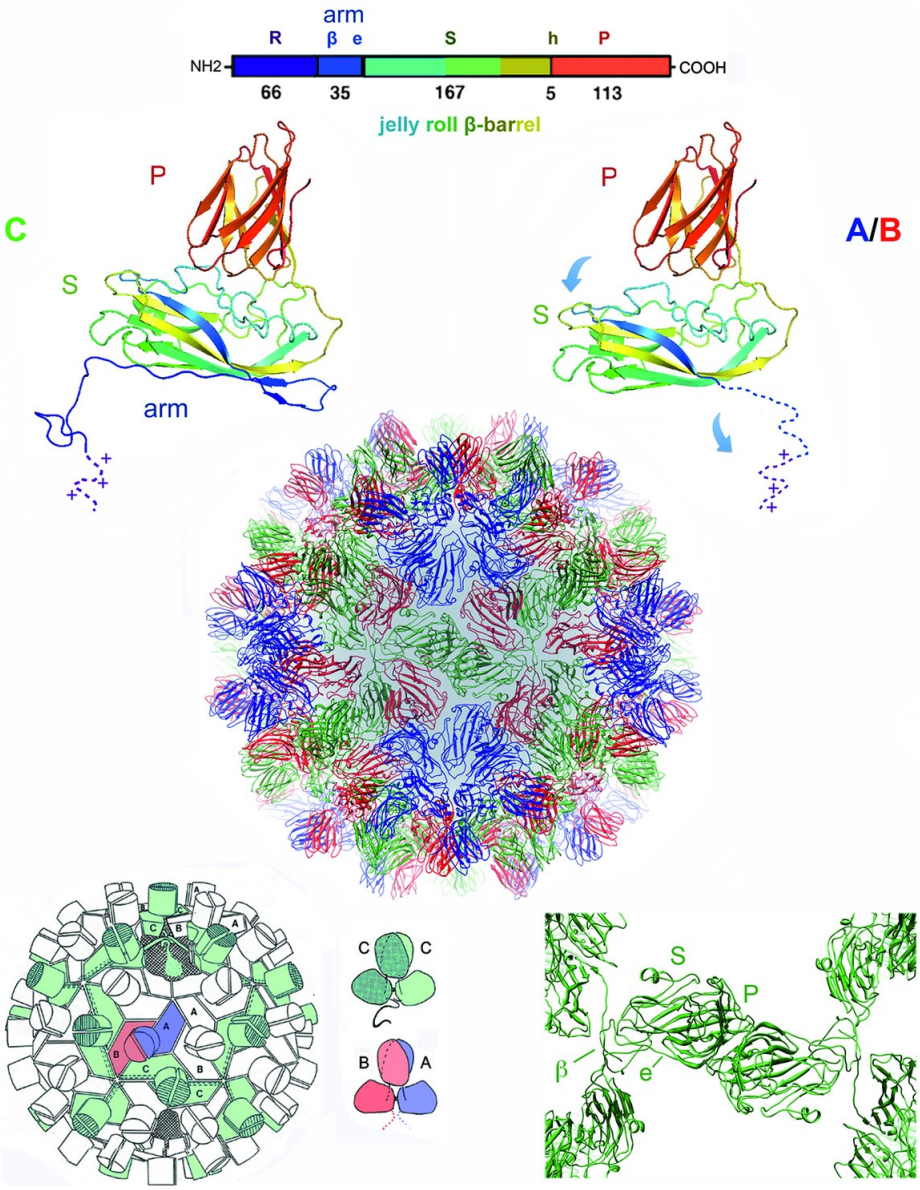

FIGURE 3.9 **Tomato bushy stunt virus (TBSV), a T = 3 icosahedral structure**. *Top*: Modular organization of the TBSV coat protein polypeptide chain. R: unstructured, positively charged N-terminal region. β, e: segments of the "arm," ordered on the C-conformation subunits and unstructured on the A- and B-conformation subunits; when ordered, the β segment forms an interdigitated "β-annulus" with corresponding segments from two other chains, and the e segment extends along the base of the subunit (see panel at *bottom*, *left*). S: shell domain, a β-jelly roll. P: projecting domain, a β-sandwich of somewhat different fold from the jelly-roll S-domain. h: hinge between the S and P domains. The color coding in the bar representation of the chain is repeated in the ribbon diagrams of the C (*left*) and A/B (*right*) conformations. Note that the two conformations differ in two respects: the ordering of the arm and the hinge angle between S and P domains (*curved arrows* on the right-hand ribbon diagram). *Center*: Ribbon representation of the entire protein coat of the virus; the colors of the A-, B-, and C-conformation subunits are as in Figure 3.6. *Bottom left*: Schematic figure, showing that the arms of the C-subunits (*green*) interdigitate around threefold axes of the icosahedral symmetry, forming a coherent inner framework. *Bottom right*: Magnified view of some of the C-subunits from the coat seen in the central part of the figure, illustrating the β-annulus (β) and the extended part of the arm (e). In the bottom center are schematic views of the C-C and A-B dimers, showing how the hinge between S and P domains correlates with the ordering of the arms. When ordered, the arms fit into the slot between S domains, which have rotated away from the contact that they have when the arms are unfolded into the particle interior).

How equivalent or nonequivalent are the actual intersubunit contacts in TBSV and related structures? Most of the interfaces are well conserved, with very modest local distortions that do not significantly change the way individual amino acid side chains contact each other. The interfaces between conformers that do exhibit noteworthy differences are those that include the ordered arms in one of the quasi-equivalent locations (the C-conformer). At these interfaces, there is a discrete switch between two states, with ordering and disordering of the arm as the toggle. Nonetheless, many side chain contacts are conserved around the "fulcrum" that relates an A/B dimer to a C/C dimer (Fig. 3.9).

Only certain multiples of 60 subunits can pack with the kind of quasi-equivalent contacts illustrated by the commas in Figure 3.6; these multiples are given by the formula $T = h^2 + hk + k^2$, where h and k are any integer or zero.[59] The multiple T is known as the "triangulation number," because, as illustrated by comparison of the 60- and 180-comma structures in Figures 3.4 and 3.6, they correspond to subtriangulations of an icosahedral net on the surface of a sphere. Such nets are known as "surface lattices." If we think of an icosahedrally symmetric structure as a folded-up hexagonal net (Fig. 3.10), then 12 uniformly spaced sixfold vertices are transformed into fivefold vertices.

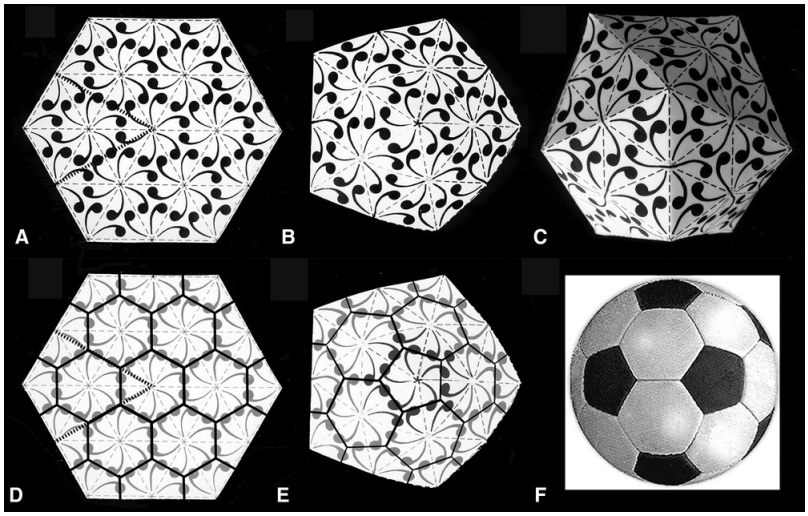

FIGURE 3.10 **Generation of curved structures from planar lattices**. **A**. Portion of a hexagonal lattice. Six triangular cells of the lattice meet at each lattice point, and each triangular cell contains 3 "subunits" (commas). Thus, there is a sixfold symmetry axis at each lattice point, a threefold symmetry axis at the center of each triangle, and a twofold axis at the midpoint of each edge. Imagine that the lattice extends indefinitely in all directions. **B**. Curvature can be introduced by transforming one of the sixfold positions into a fivefold (*center*). A 60° "pie slice" has been removed from the object in **(A)** by cutting along the *heavy dotted lines*, and the cut edges have been joined to generate the curved lattice shown here. **C**. If further cuts are made at regular intervals in an extended lattice, such as the one in **(A)**, and the edges joined as in **(B)**, a closed solid can be produced. In the case of the icosahedral solid shown here, vertices of the lattice separated by two cell edges have been transformed into fivefolds, while the intervening lattice points have been left as local sixfolds, producing a T = 4 (h = 2, k = 0) structure. Notice that the local sixfolds are actually only approximately sixfold in character; they correspond strictly to the twofold axes of the icosahedral object. **D**. Lines joining the centers of the triangular cells in **(A)** create a pattern of hexagons. **E**. When a sixfold is transformed into a fivefold, a hexagon becomes a pentagon. **F**. If second nearest-neighbor lattice points are all transformed into pentagons, a soccer-ball figure results. This is a T = 3 structure. A description of the lattice as a network of hexagons and pentagons is complementary to its description as a network of triangles. The representations in Figures 3.4B and C, 3.6, 3.7B and E, 3.10A-C, and 3.17A use triangles. The representations in Figures 3.10D-F and 3.17B use hexagons and pentagons. One representation for a given lattice can be derived easily from the other (compare Figs. 3.10A with 3.10D and 3.10B with 3.10E).

Other Types of Icosahedral Surface Organization

Hexagonal packing is an efficient way to "tile" a surface (think of hexagonal floor tiles), even if the building blocks themselves do not have sixfold symmetry. In many larger, icosahedrally symmetric virus particles, the outer-shell building blocks ("assembly units") are centered at the vertices of an icosahedral surface lattice, subtriangulated with one of the allowed T numbers, but the oligomeric building blocks themselves are not hexamers. In adenoviruses (Fig. 3.11), they are trimers, but with two similar β-barrel domains and a roughly hexagonal outline; a chemically distinct, pentameric building block, with a single β-barrel domain per subunit occupies the fivefold vertices. In the polyoma- and papillomaviruses, the assembly units are all identical pentamers (Fig. 3.1). Viewed at low resolution (eg, by negative-stain electron microscopy), all these viruses have globular "lumps" at the vertices of a lattice with one of the allowed triangulation numbers (T = 25 for the adenoviruses: Figure 3.11; T=7 for the polyoma- and papillomaviruses: Fig. 3.1), but when seen at higher resolution, the six-coordinated lumps are actually trimers or pentamers, respectively. The next section describes mechanisms by which assembly of these units conserves specificity of local interactions, an essential characteristic for well-defined molecular association in biology. The globular lumps seen by low-resolution electron microscopy were originally called "capsomeres" (meaning the "structural units" of the "capsid"); the word is still used when referring to apparent morphological units on the surface of a virus shell, particular for cases in which all capsomeres are the same and represent a defined oligomer, as in the pentameric units of polyoma- and papillomaviruses.

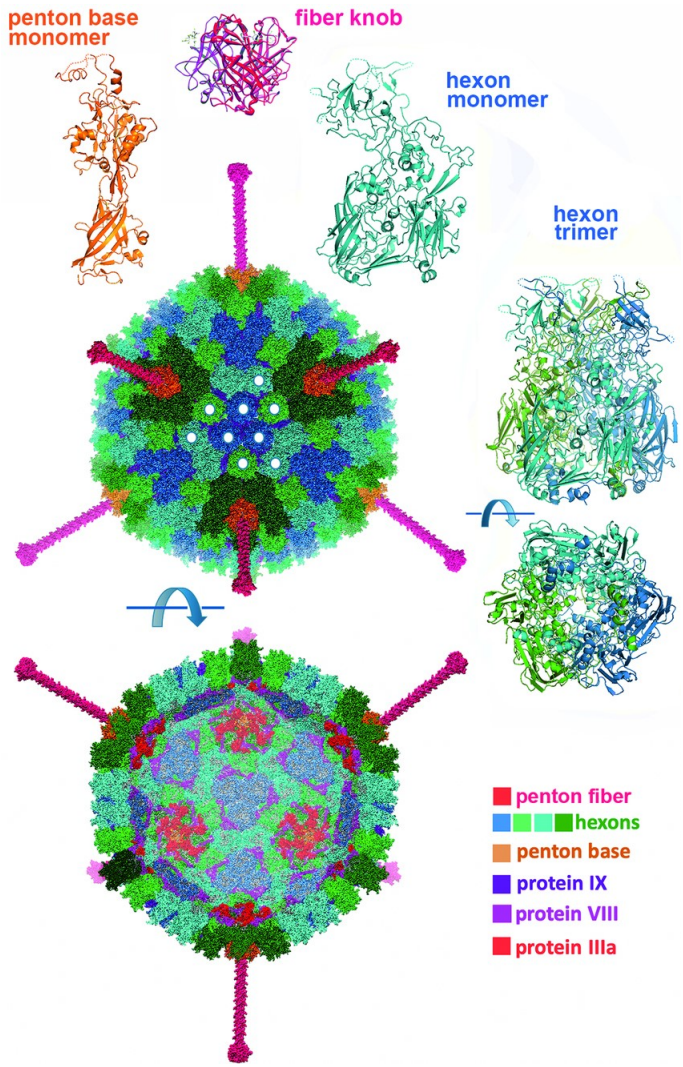

FIGURE 3.11 **Adenovirus capsid structure**. The principal building blocks—hexon, penton base, and penton fiber (trimeric rod with terminal knob)—are held together by "cement proteins" (pIIIa, pVIII, and pIX). The large images are an outside view of an adenovirus type 5 particle (*upper*) and a cutaway (note flip shown by *curved arrow*), showing the internal surface. The coiled DNA that fills the interior and proteins closely associated with it are not shown. The hexon monomer has two similar jelly-roll β-barrels; the trimer is, therefore, a pseudo-hexamer of those barrels. Note how an N-terminal extension of the monomer reaches under one of the two other subunits and how the "tower" of loops, which project to the outer surface of the virion, twists around so that the subunits intertwine tightly. Variation of these loops creates the principal antigenic differences among adenovirus serotypes. Each "group-of-nine" hexons (*white circles*, outside view) defines an icosahedral face. The cement protein, pIX (*purple*), inserts into grooves between hexons on the outer surface of the particle and holds a group of nine together; twenty such groups of nine make up most of the shell. Twelve "groups of six" (one penton base subunit, *orange-brown*, and five "peripentonal" hexons, *dark green*) fill the remaining gaps at each icosahedral fivefold position. The inside view shows the locations of pIIIA (*red*) and pVIII (*magenta*), which line the inner face of the shell at defined positions around the inward facing ends of the groups of six (really, 5 + 1) and the cluster of hexons (*dark blue*) at particle threefold axes, respectively. (Particle images courtesy of Z.H. Zhou: Liu H, Jin L, Koh SB, et al. Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science*. 2010;329:1038-1043; Dai X, Wu L, Sun R, Zhou ZH. Atomic structures of minor proteins VI and VII in human adenovirus. *J Virol*. 2017;91. Refs.[92, 236]). Subunit cartoons are from PDB 1X9P (penton base of adenovirus type 2) (Zubieta C, Schoehn G, Chroboczek J, Cusack S. The structure of the human adenovirus 2 penton. *Mol Cell*. 2005;17:121-135. Ref.[445]), 3EXW (fiber knob of adenovirus type 7) (Persson BD, Muller S, Reiter DM, et al. An arginine switch in the species B adenovirus knob determines high-affinity engagement of cellular receptor CD46. *J Virol*. 2009;83:673-686. Ref.[304]), and 1P30 (hexon of adenovirus type 5) (Rux JJ, Kuser PR, Burnett RM. Structural and phylogenetic analysis of adenovirus hexons by use of high-resolution x-ray crystallographic, molecular modeling, and sequence-based methods. *J Virol*. 2003;77:9553-9566. Ref.[342])

The flaviviruses illustrate yet another adaptation to icosahedral packing. The asymmetric unit of an icosahedral surface lattice can be represented by a (spherical) triangle with a fivefold axis and two adjacent threefold axes as its vertices (see Fig. 3.10). The flavivirus envelope protein (E) is a flat, elongated dimer[326]; three such dimers neatly fill a twofold-related pair of asymmetric-unit triangles, with the dyad of the central dimer coincident with the icosahedral twofold (Fig. 3.12).[215] The shell contains 180 subunits, but not in a T = 3 arrangement. Instead, there is a herringbone- or raftlike packing, with one dimer centered on an icosahedral twofold axis and a second dimer incorporated parallel to it, with only a local twofold axis relating its two subunits.

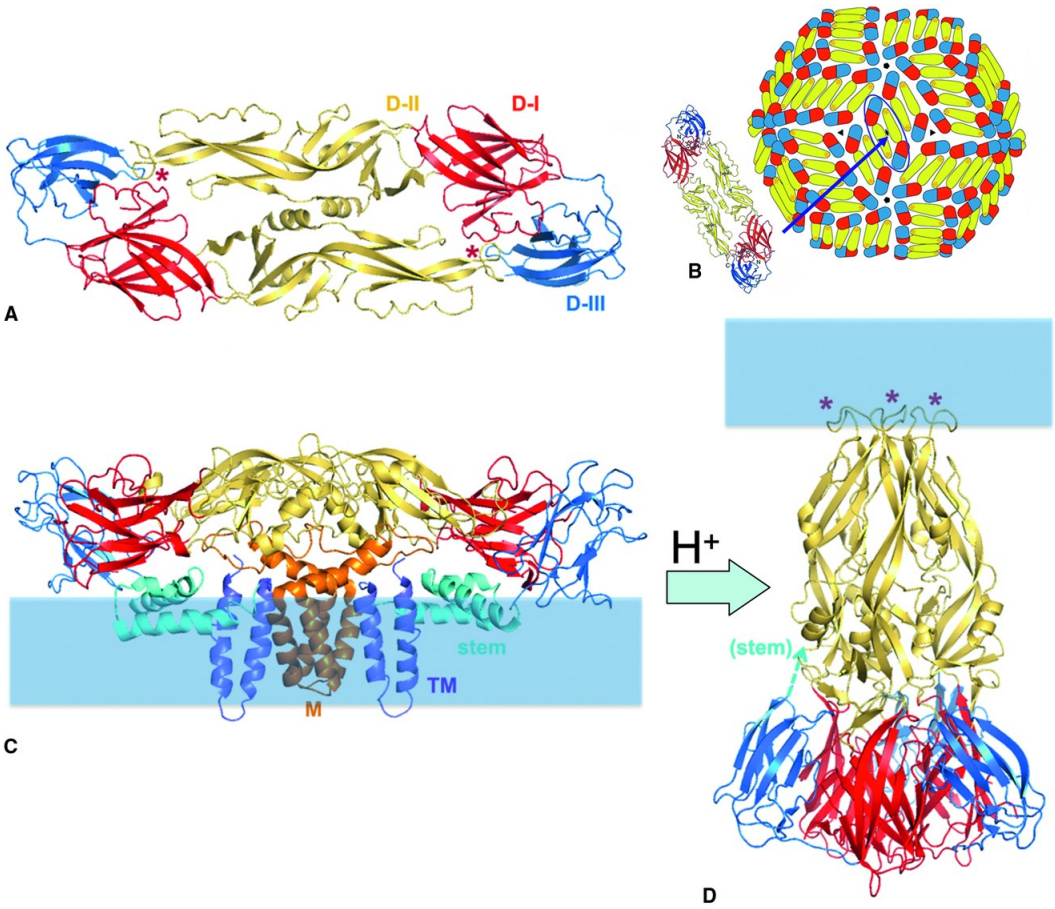

FIGURE 3.12 **Flavivirus structure**. **A**. A dimer of the dengue virus E-protein ectodomain in the conformation found on mature virions, viewed from outside the virus particle. Each subunit folds into three domains: a central domain I (*red*), an elongated domain II (*yellow*), which bears the fusion loop at its tip (*orange asterisk*), and an Ig-fold domain III (*blue*). Domain II folds from two long, disulfide-stabilized loops that emanate from domain I. The domain coloring is consistent throughout the figure. **B**. Packing of 90 E dimers on the surface of a mature virion. **C**. "Side" view of a full-length E dimer and an associated M dimer (*gold*), from a cryo-EM structure of a dengue virion (ref). The segment ("stem": *cyan*) that connects domain III with the transmembrane hairpin (TM: teal) is an amphipathic, three-helix bundle, lying at the hydrophilic-hydrophobic interface of the membrane bilayer (semitransparent *gray-blue*). **D**. Postfusion E trimer, from a crystal structure of the dengue virus E ectodomain. Other structures confirm that the polypeptide chain extends along domain III and that the distal part of the stem and the TM hairpin interact with the fused membrane. The fusion loops (*asterisks*) cluster to present a hydrophobic surface.

The arrangement of 120 copies of the inner- (core-) shell protein in dsRNA viruses is example of completely nonequivalent packing of two identical copies of a protein subunit (Fig. 3.13). There are two fully distinct environments for this protein (designated A and B in Fig. 3.13, lower right). The amino acid side chains on the lateral surface of the core-shell protein have different partners, depending on the interface in which they lie. The distortion of the subunit itself, when the VP2 subunits at A and B positions are compared, is quite small.

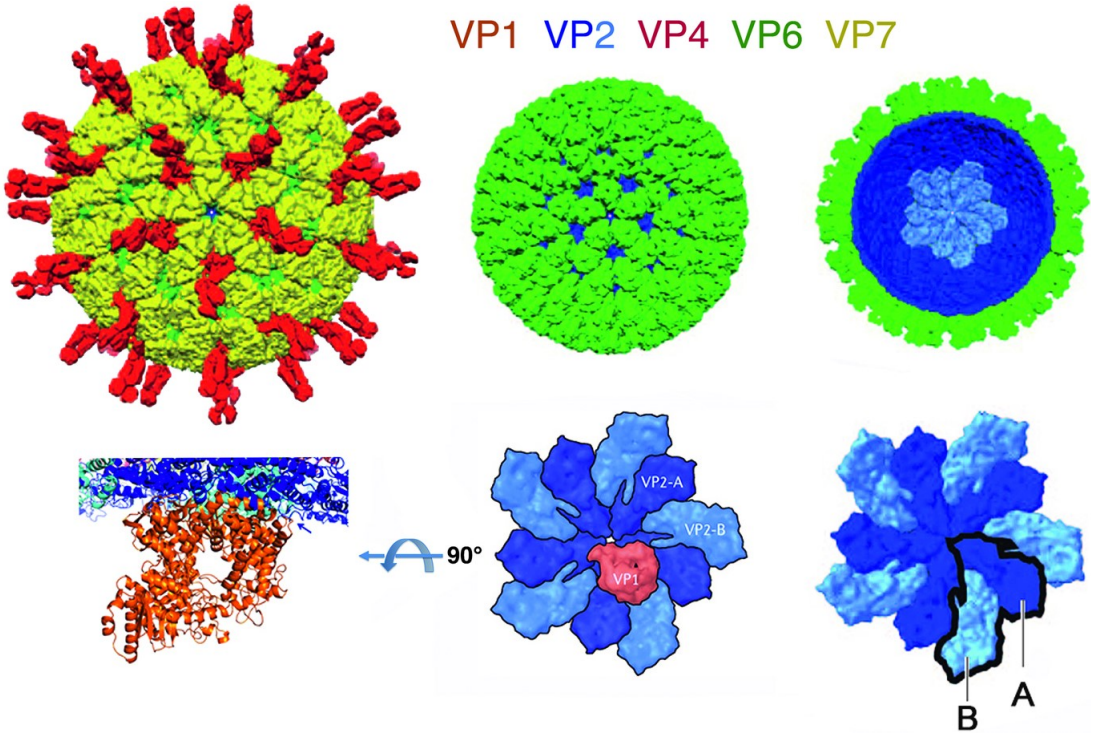

FIGURE 3.13 **Rotavirus**. Subunits colored as in the key at the top. **Top row**, *left to right*: Surface views of the triple-layer particle (TLP); double-layer particle (DLP); and DLP cutaway across the middle of the particle, showing an inside view of the VP2 layer. One VP2 decamer is in a lighter color. All views are along a fivefold axis. The eleven segments of the dsRNA genome (not shown here) fill the cavity of the particle. **Bottom row**, *right*: Magnified view of the central VP2 decamer in the cutaway view above it. The two VP2 conformers, designated A and B, are now in *dark blue* and *light blue*, respectively. The platelike subunit has a crudely triangular shape as it tapers toward the fivefold axis. A solid black border outlines one A-B pair. Note that at the A:B interface within this pair, the A subunit contacts B along a surface of the latter proximal to the apex of the triangle, whereas at the B:A interface across the *black line*, the corresponding part of the B subunit contacts a surface on A distal to the apex. That is, the contacts are completely nonequivalent. **Bottom row**, *center and left*: Position of a single VP1 (polymerase and capping enzyme) with respect to a VP2 decamer. In the tangential view on the left, the subunits are in "cartoon" representation.

Frameworks and Scaffolds

Accuracy of assembly is critical for efficient production of infectious particles. The protein subunits of TBSV or picornaviruses have extended N- or C-terminal arms augmenting a central jelly-roll β-barrel. These arms form an internal framework, such as the one illustrated for TBSV in Figure 3.9. The TBSV "assembly unit"—the oligomer of the coat subunit that forms spontaneously in solution (and by inference, in the cell following its synthesis)—is a dimer, which can have two conformations—an "A/B" dimer, with disordered N-terminal arms, and a "C/C" dimer, with folded arms.[159] The local curvature of those two conformations is different, and the framework of C/C arms fixes the overall diameter of the particle. Removal of the N-terminal arms of TBSV-like subunits leads to self-assembly of a small, 60-subunit icosahedrally symmetric particle that cannot package RNA.[136] That is, without the arms, there is no mechanism for a conformational switch. Moreover, the way the C/C arms link at threefold axes creates a framework that requires pentamers of A/B dimers to fill the gaps around fivefold axes. Related mechanisms, with variations, regulate assembly of caliciviruses.[71,201,313]

In the papilloma- and polyomaviruses, N- and C-terminal extensions (principally the latter) of the subunit globular domains tie together the pentameric building blocks, which have almost no contacts except through these extensions (Fig. 3.14).[232,365,366,415] Flexibility of the arms allows formation of the different kinds of contacts required to surround a pentamer with six other pentamers (ie, to position a pentamer at the 6- as well as at the 5-coordinated vertices of a T = 7 subtriangulated icosahedral lattice). The C-terminal arms emanate from one pentamer and dock into another. The way they dock is the same for all 360 arms, with identical interactions locking them in place, but not their configuration between the point at which they emerge from the globular domain of their subunit of origin and the point at which they dock into their target subunit. This is a different sort of "quasi" equivalence than the one described by Caspar and Klug.[59] All the nonequivalence is incorporated into a short segment of the C-terminal arm as it traverses from its pentamer of origin to its docking site in its target pentamer.[365]

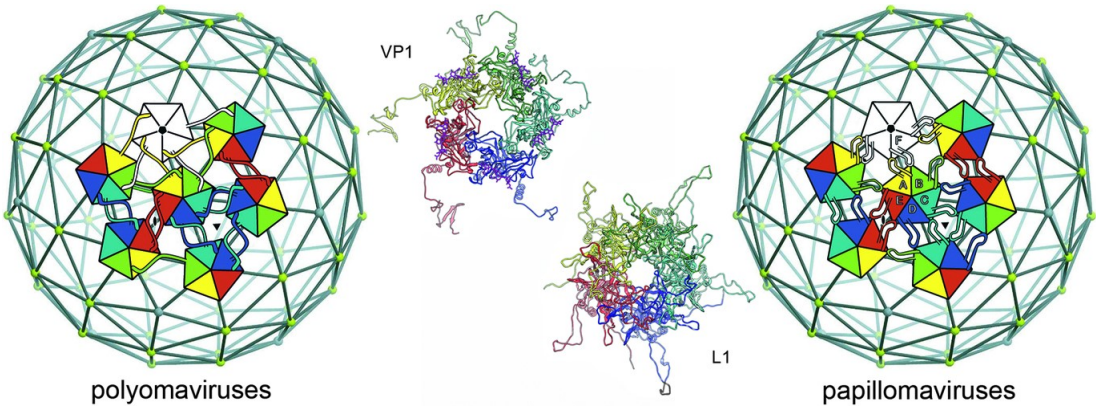

FIGURE 3.14 **Packing of pentamers in the capsids of polyoma- and papillomaviruses**. The ribbon diagrams in the center show pentamers of VP1 (polyomaviruses) and L1 (papillomaviruses), viewed from their outward-facing surfaces. Note the C-terminal arms of the subunits, which extend away from the pentamers in VP1 but loop back to it in L1. The schematic diagrams to the *left and right* illustrate the packing of these pentamers in the virion shell. The framework shows a T = 7 icosahedral lattice; VP1 or L1 pentamers are centered on both 6- and 5-coordinated positions.

Larger and more complex structures, such as adenoviruses (Fig. 3.11), have separate framework proteins, rather than using long extensions emanating from the folded domain(s) of a subunit.[218,236,322] The principal outer shell components of adenoviruses are "hexons," trimers of a subunit with two, similar jelly-roll domains[328] and "pentons," pentamers of a subunit with a single jelly-roll β-barrel domain.[445] If all the jelly-roll β-barrels were the same, the structure would correspond to T = 25 quasi-equivalence, just as if all the picornavirus jelly-roll β-barrels were the same, the particles would be T = 3 shells, like TBSV and its plant-virus relatives. A set of additional proteins cements the adenovirus structure together and determines its size (Fig. 3.11).[92,128,236,367,368] The elaborate interaction patterns of these "cement" proteins stabilize a "group of nine" hexons, centered on the icosahedral threefold axis, and a "group of six" (five hexons surrounding a penton), centered on the icosahedral fivefold axis.[223,236] The structures of adenovirus-like bacteriophages, PRD1[26] and PR772,[320] show a somewhat simpler size-determining and stabilizing framework: a "tape-measure protein" extends from the penton toward the icosahedral twofold axis, where it interacts with an identical protein running toward it from the twofold related penton (Fig. 3.15).[3] Unlike adenoviruses, the adenovirus-like bacteriophages have a lipid-bilayer membrane between the P3 layer and the internally coiled DNA.[10,17,78]

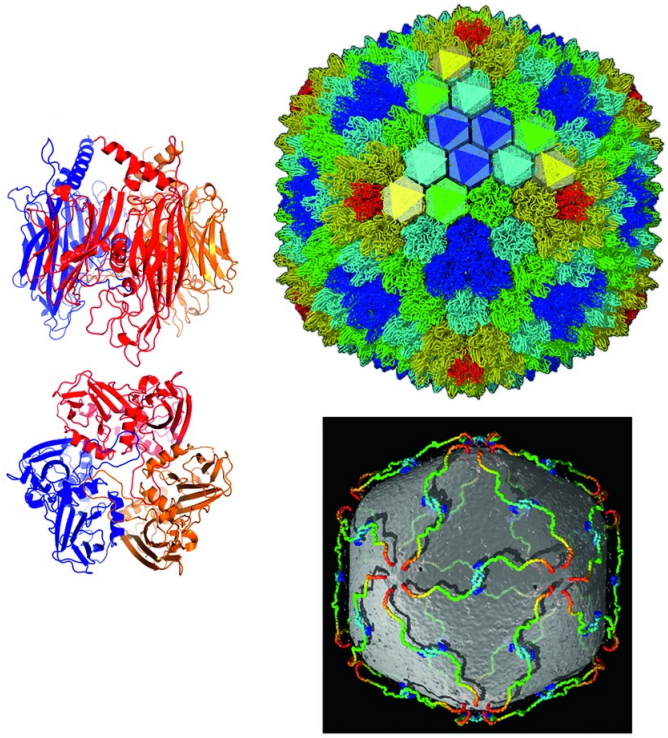

FIGURE 3.15 **Bacteriophage PRD1**. *Left*: Side and bottom views of the "hexon" protein, P3. The colors correspond to those in the ribbon diagrams of the adenovirus hexon trimer in Figure 3.11. Like the adenovirus hexon, P3 has two jelly-roll β-barrels, but the loops that project outward are much less elaborate (Courtesy of David I. Stuart, Oxford University. [Please cite the existing credit lines as references (Kindly refer to manuscript, prev. ed.). Ref.[26]). The image on the *upper right*, based on a crystal structure of the intact phage particle, is a view along a twofold axis. One threefold set of P3 trimers is highlighted by triangles. The pentons (P31) are in *red*. At the *lower right* is a view with the outer layer stripped away, to show the extended "tape-measure" protein, P30, which helps determine the size of the shell, and the lipid bilayer just beneath it. There are 60 copies of P30; each chain extends from a twofold axis (N-terminal end, *blue*) to the inner surface of a penton (C-terminal end, *red*). At the twofold axis, one P30 associates with a second, twofold-related P30.

During assembly of the heads of most double-stranded DNA bacteriophages, an internal scaffold protein directs formation of an empty precursor known as a "prohead."[54,123] Assembly of proheads that can package DNA often nucleates around scaffold components associated with the dodecameric "portal protein" situated at the unique vertex through which DNA will enter and leave and to which the tail structure will attach after DNA filling is complete.[98] Conformational signals related to DNA packaging trigger release and recycling (eg, P22) or degradation (eg, T4) of the scaffold, accompanied by a reorganization and expansion of the head (Fig. 3.16A-D).[114,199] In bacteriophage HK97, the scaffold is an N-terminal extension of the T = 7 capsid subunit, cleaved from the rest of the protein in the transition from prohead to head.[82,168,180,222,409] In dsDNA bacteriophages, and in adeno- and herpesviruses, DNA is pumped into the empty head, through the portal protein, until it reaches the tightly coiled state illustrated in Figure 3.16E.[115,116,363]

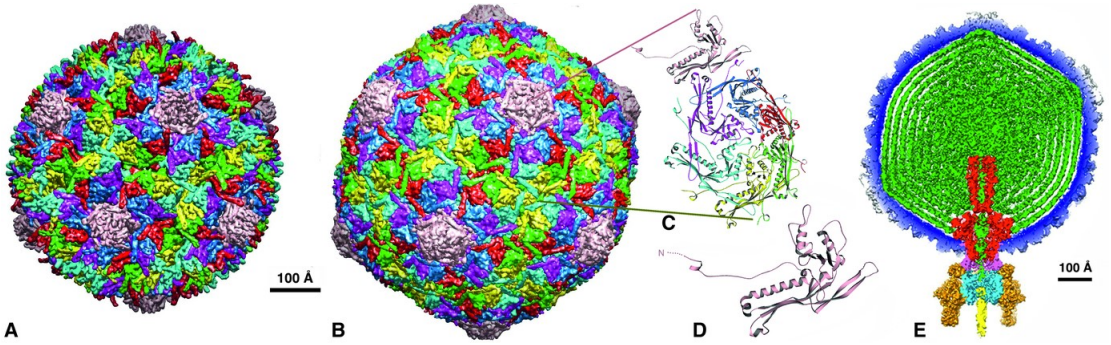

FIGURE 3.16 **Capsid reorganization and DNA packaging in tailed bacteriophages**.[199] **A**. Surface of the HK97 procapsid. The surface organization is a locally distorted T = 7 arrangement, with fivefold symmetric association of the subunit at the fivefold positions (beige) but a skewed arrangement in the rings of six subunits that surround a local six-coordinated position (colored in *magenta*, *blue*, *red*, *green*, *yellow*, *cyan*, in clockwise order).[82] An N-terminal extension of the head subunit is the scaffold for prohead assembly; its cleavage by a coassembled protease triggers rearrangement of the subunits into the expanded, thinner, more angular shell illustrated in panel **(B)**.[111] **B**. Capsid (head) of the mature HK97 particle; molecular surface, based on crystallographic model, colored as in A.[409] This view is oriented so that a fivefold axis is vertical. The image is derived from the structure of an empty capsid with 420 subunits in a T = 7 icosahedral lattice. In a wild-type bacteriophage particle, a portal protein connected to a tail replaces one of the rings of five subunits (see panel **E**). **C**. Expanded view in ribbon representation of one icosahedral asymmetric unit (ie, one of the five subunits in the pentameric ring and one each of the quasi-equivalent subunits in the hexameric ring). All subunits are chemically identical. In HK97, but not in many related bacteriophages, an intersubunit isopeptide bond, which forms during maturation, cross-links the entire coat.[111] **D**. A further enlarged view of a single, 31 kDa subunit. The 105-residue N-terminal extension that functions as an assembly scaffold is indicated schematically by a *dotted line*. **E**. Cutaway representation of a three-dimensional cryo-EM image reconstruction of bacteriophage P22. Its assembly is formally similar to that of HK97, but there is a distinct, recycled scaffold protein[54] and no covalent cross-linking of the head.[298] The packaged DNA (*green*) winds tightly around an internal extension of the portal protein (*red*).[286,378] (Images in **A–D** from VIrus Particle Explorer [VIPERdb] Website, http://viperdb.scripps.edu/. Ref.[321])

Mass Production and Specificity

A fundamental principle embodied in all the various structures just described is one of mass production. One or more standard building blocks assemble into the larger structure. In simple (T = 1) cases, such as the parvoviruses and picornaviruses, a repeating set of identical interactions determines the final structure. In the T = 3 structures (eg, many plant viruses; caliciviruses), the building block is a subunit dimer, which has two, scaffold-regulated states. In polyoma- and papillomaviruses, the building block is a pentamer of the major capsid protein, again with two states (penta- and hexa-coordinated), probably switched by interpentamer contact of the C-terminal arms.[365,366] In more elaborate cases, such as adenoviruses, the basic subassembly building blocks are hexons and pentons, and the groups of nine and six may represent further assembly intermediates, stabilized by modulators (eg, the minor "cement" proteins) to ensure the correct relative placement of hexons and pentons and a well-determined overall outcome.[323,345,425] Hierarchies of subassemblies characterize very complex particles, such as tailed bacteriophages. Head assembly and tail assembly are separate pathways, and only fully assembled, DNA-filled heads and fully assembled tails then join, ensuring that an error in one subassembly does not waste components and energy that went into the other.[54] Budding of many enveloped viruses, with distinct subassemblies for the internal and external components, embodies a similar principle.

Elongated Shells

The examples in Figure 3.17 illustrate elongated particles with caps at either end. In many of the dsDNA bacteriophages, the shell looks like a familiar icosahedral design at the poles. As the lattice approaches the equator, however, the regular interspersion of fivefolds and local sixfolds gives way to local sixfolds only, so that there is a tubular region around the middle of the particle (Fig. 3.17A).[380] The tubular region can be of varying extent; in extreme cases, it can be much longer than the caps themselves. A "Q number," analogous to the T number, describes both the extent of the equatorial tube and the rotational offset of the two, hemi-icosahedral caps.[123,266] A further variation on this theme is found in the shells formed by the CA fragment of the lentivirus Gag protein. Conical structures seen within HIV-1 particles (Fig. 3.3D) have been shown to be based on the sort of arrangement shown in Figure 3.17B, where one cap has more than 6 fivefold positions and the other has less, so that the diameters of the two caps are different.[131,132,253] (Note that if there are only sixfold and fivefold vertices in a closed surface lattice, there will always be exactly 12 of the latter.)

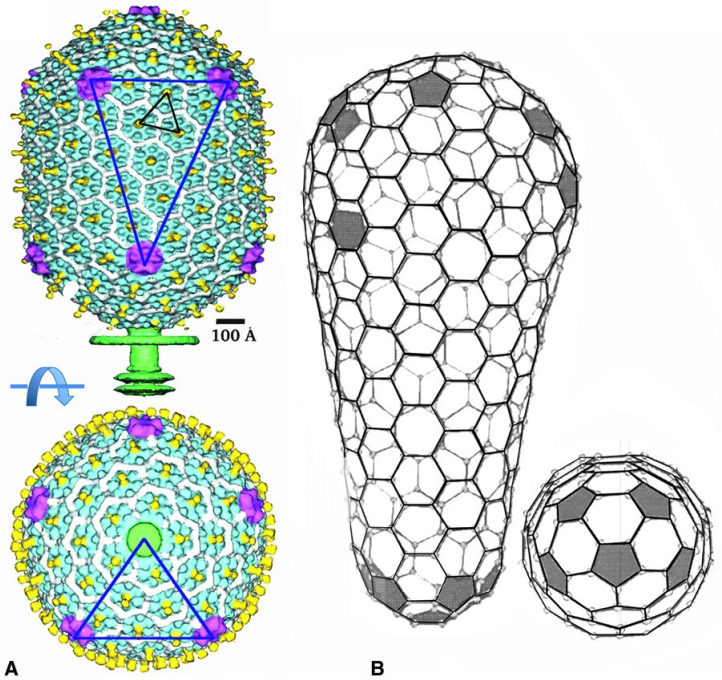

FIGURE 3.17 **Elongated shells with icosahedral caps**. **A**. Surface of a mature bacteriophage T4 head, viewed normal to the principal axis (*top*) and along the principal axis from the tail (*bottom*). The principal axis is a fivefold axis of the top and bottom caps, which are hemi-icosahedra with a T = 13l lattice. The processed major capsid protein, p23*, is in *blue*; the processed fivefold protein, p24*, in *magenta*; accessory proteins, somewhat similar in function to the adenovirus "cement" proteins, are soc, in *yellow*, and hoc, in *light gray*. The proximal segment of the tail is in *green*. An equatorial band of p23* hexamers elongates the structure: the larger, skewed triangle joins three p24* pentamers that would otherwise be related by an icosahedral threefold axis (*smaller triangle*). (Modified from Fokine A, Rossmann MG. Molecular architecture of tailed double-stranded DNA phages. *Bacteriophage*. 2014;4(1):e28281. Copyright © 2014 Landes Bioscience. Reprinted by permission of Informa UK Limited, trading as Taylor & Francis Group, http://www.tandfonline.com. Ref.[123]) **B**. Diagrammatic representation of an HIV-1 capsid surface lattice. Imagine each hexagon and pentagon to contain six and five CA subunits, respectively. Seven sets of locally fivefold-related subunits (*shaded pentagon*) in the upper cap and five sets in the lower one, connected by bands of sixfold-related CA subunits, generate a tapered, conelike shell. On the lower right is a view of the smaller tip of this particular capsid lattice. Note that the (local) fivefolds are not uniformly spaced, and that neither cap has overall fivefold symmetry. (Adapted from Ganser BK, Li S, Klishko VY, et al. Assembly and analysis of conical models for the HIV-1 core. *Science*. 1999;283(5398):80-83. Reprinted with permission from AAAS. Ref.[131])

Multishell Particles

Most dsRNA viruses have a genuinely multishell icosahedral organization, with some common features and some variation from group to group (see Chapters 11-13, volume 3). In virions of the mammalian dsRNA virus groups (reoviruses, rotaviruses, and orbiviruses), the innermost protein shell contains 120 copies of a large, platelike protein[145,254,324] (Figs. 3.13 and 3.18). Surrounding the inner shell is a second characteristic layer, containing, in most cases, 780 copies of a trimeric protein with a radially directed jelly-roll β-barrel and inwardly directed N- and C-termini, which together form a largely α-helical "base" domain.[144,233,251] This second layer corresponds closely to a "classical," quasi-equivalently packed, T = 13 icosahedral shell—all the interactions between adjacent trimers are variations on the same set of contacts.

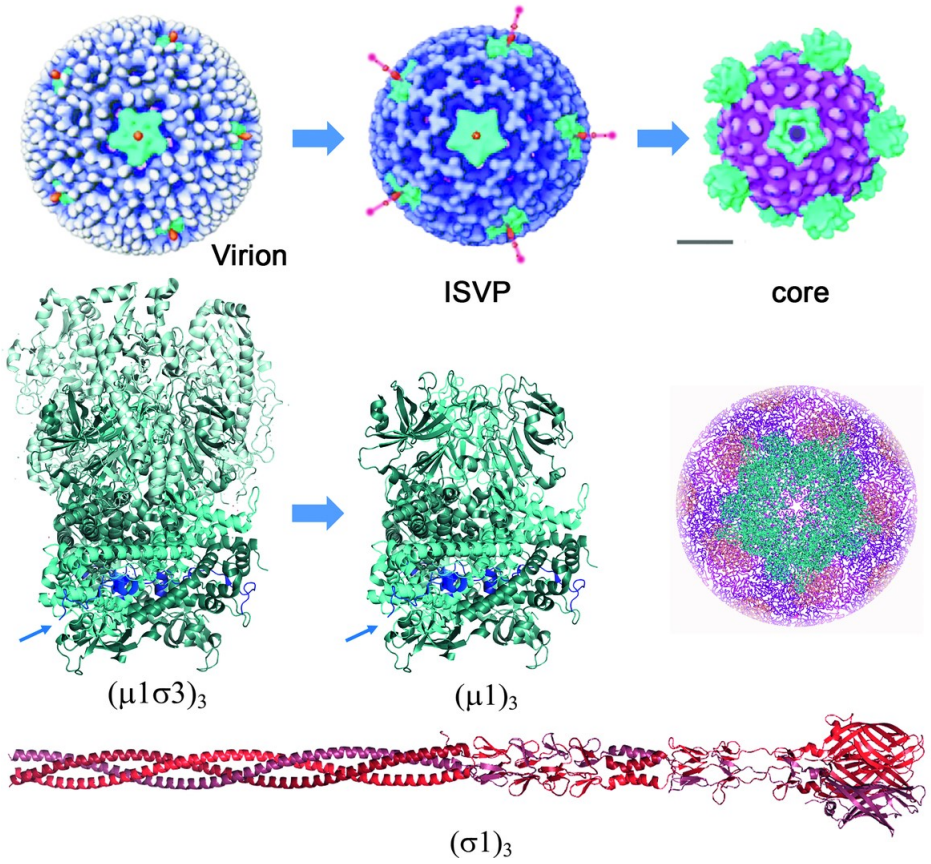

FIGURE 3.18 **Reovirus structure and transitions during reovirus entry**. *Top row*: Surface renderings of reovirus particle (*left*), infectious subviral particle (ISVP: *center*), and core (*right*). The pentameric turrets of λ2, the RNA capping protein, project through the outer shell, a fenestrated, T = 3 laevo, array of μ1σ3 heterohexamers. Proteolysis (*first large arrow*), either by cathepsins in late endosomes or by proteases in the gut, removes σ3 and stiffens the trimeric, receptor-binding spikes of σ1 (*bottom row*[104,325]), which project from the λ2 turrets. The knobs at the tip of the spikes bind receptor. Scale bar = 200 Å. (Figure adapted from Dryden KA, Wang G, Yeager M, et al. Early steps in reovirus infection are associated with dramatic changes in supramolecular structure and protein conformation: analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction. *J Cell Biol*. 1993;122:1023-1041. Ref.[109]) *Middle row*: Backbone cartoons of $(\mu1\sigma3)_3$ (*left*) and $(\mu1)_3$ (*center*). The σ3 subunits are in *light blue-gray* shades; μ1 subunits are in *cyan* and two darker, related shades. The first 42 residues, myr-μ1N, separated from the rest of μ1 by autolytic cleavage, are in contrasting shades of *blue*. *Small arrow* marks the first ordered residue in the structure, near the N-terminus of μ1N; the myristoyl group may insert back into a hydrophobic slot just above the position at which the N-terminus emerges.[233,438] μ1N, released from the receptor-bound ISVP, forms membrane pores, apparently facilitating release of the core particle into the cytosol; the specific, molecular trigger of μ1N release is not known. Middle row (right): Close-up of the λ2 turret (from PDB:1EJ6). *Bottom row*: σ1 spike in cartoon representation. The three identical subunits are in contrasting shades of red. Composite of PDB:6GAP and PDB:3S6X. See Ref.[104].

Various elaborations and simplifications of the two-layer design just described differentiate the families of dsRNA viruses. For example, in the orthoreoviruses (Fig. 3.18), the T = 13 layer has gaps, through which pentameric "turrets" of yet another protein, anchored on the inner shell, project; only 600 of the potential 780 subunits are present.[46,109,324] The birnaviruses lack the 120-subunit layer altogether and have instead 780 copies of a single major capsid protein, with a shell domain that resembles those of plant and insect viruses and a trimer-clustered projecting domain that resembles the jelly-roll β-barrel in the T = 13 shell of rotaviruses and orbiviruses.[83] The T = 13 packing of the shell domain so closely recalls that of its counterparts in T = 3 and T = 4 positive-strand RNA virus structures that an evolutionary common origin of those domains seems plausible. Similarities in the RNA-dependent RNA polymerases of these viruses also suggest some shared ancestry.[293] The dsRNA bacteriophages such as φ6 contain the 120-subunit, inner shell layer and a fenestrated, T = 13 layer (rather like reoviruses), enclosed within a lipid bilayer membrane.[46,181,190,401]

Rearrangements in Surface Lattices

Icosahedral surface lattices can undergo rearrangements, which preserve the overall symmetry of the structure but change the pattern of specific intersubunit contacts. There can be an accompanying change in the diameter of the shell. These rearrangements are cooperative—that is, they occur more or less simultaneously across the whole structure. They are also "displacive"—that is, they do not require dissociation and reassociation but are instead smooth transitions from one end state to another. When dsDNA bacteriophages such as P22 insert their genomic DNA into a preformed prohead, the outer shell of the prohead expands as its subunits shift around to form the mature structure[54,82,196,199,222] (Fig. 3.16). Another well-characterized example is expansion of the T = 3 plant viruses, which occurs when the calcium ions that stabilize a particular set of subunit interfaces are removed[16,329] (Fig. 3.19). This swelling is probably the first step in disassembly: plant viruses, when injected by their vectors into the cytoplasm of a recipient cell, are exposed to a low Ca^{2+} environment. A transient expansion occurs when poliovirus binds its receptor.[24] In both the T = 3 plant viruses and the picornaviruses, internally directed "arms" of the protein subunits move

outward from the interior as expansion creates gaps in the shell. Exposure of the arms may be part of the uncoating process in the case of the plant viruses or of the membrane-penetration process in the case of the picornaviruses. Cooperativity of these rearrangements implies that a few points of inhibition can prevent the change. For example, only a few intersubunit cross-links from bound neutralizing antibodies are sufficient to block infection by a picornavirus particle.[118] The same may be true of small molecules that inhibit the subunit conformational changes needed for the receptor-triggered expansion of picornaviruses.[13,307,364]

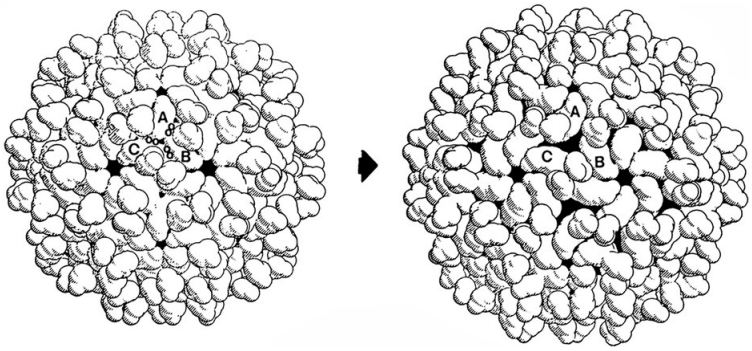

FIGURE 3.19 **Expansion of TBSV**.[329] The mature, compact particle (*upper left*) expands when Ca^{2+} ions (*small circles*) are removed. The expanded form (*upper right*) is reached by a smooth transition, in which many of the intersubunit contacts are conserved. The contacts that included the ions in the compact state have separated substantially, creating a fenestrated shell.

Helical surface lattices can also rearrange without dissociating. Contraction of bacteriophage tail sheaths is a good example (Fig. 3.20).[8,265,383] Shortening of the sheath when released from the tail plate by interactions with a bacterial cell surface is a smooth transition from one set of helical parameters to another, without gain or loss of subunits.

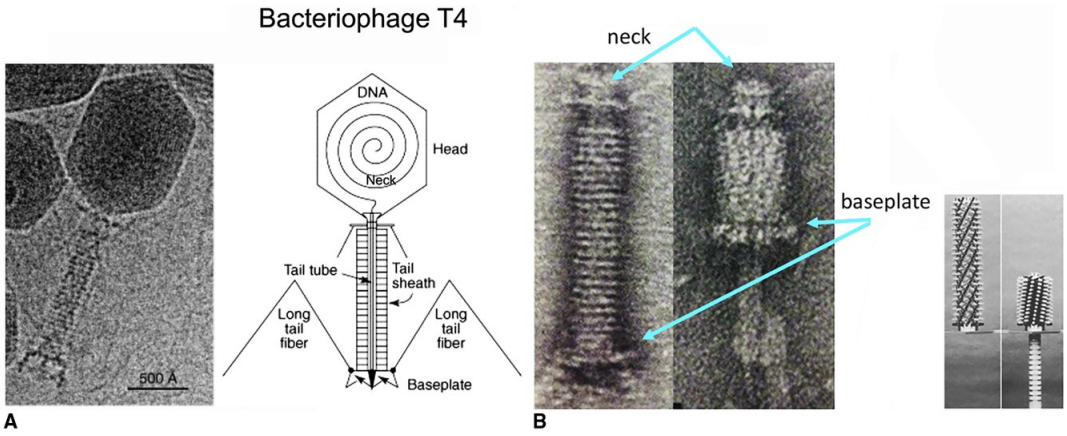

FIGURE 3.20 **Contraction of bacteriophage T4 tail sheath**. An illustration of a smooth, nondisplacive change in a structure with helical symmetry Ref.[58] **A**. Micrograph and diagram of overall structure of bacteriophage T4.[339] **B**. Surface renderings from electron micrographs of particles with extended (*left*) and contracted (*right*) tail sheaths. As the sheath contracts, it maintains its connections to the neck and baseplate, so that the baseplate—attached through tail fibers to the cell wall of the bacterium being infected—translates along the tail core, driving the tip of the core into the cell, as. (**A** reprinted from Rossmann MG, Mesyanzhinov VV, Arisaka F, et al. The bacteriophage T4 DNA injection machine. *Curr Opin Struct Biol.* 2004;14(2):171-180. Copyright © 2004 Elsevier, with permission. **B** adapted from Leiman PG, Arisaka F, van Raaij MJ, et al. Morphogenesis of the T4 tail and tail fibers. *Virol J.* 2010;7:355. http://creativecommons.org/licenses/by/2.0. Ref.[224a])

Two Recurring Globular-Domain Structures in Icosahedral Capsid Proteins

The icosahedrally symmetric shells of nearly all well-characterized, nonenveloped viruses contain one of two types of globular domain. (The known exceptions at the time of writing this chapter are the RNA bacteriophages—R17, Qβ, and their relatives[389]—and the dsRNA picobirnaviruses.[112]) One is the jelly-roll β-barrel in viruses of animals and plants, which we have described in various examples of viruses of animals and plants; it is also the principal component of icosahedral ssDNA bacteriophage capsids (eg, ϕX174).[255] The various ways this module can form a coat are quite different, and we have emphasized above the importance of framework or scaffolding components (either as extensions of the polypeptide chain of the β-barrel or as separate protein species) in directing or regulating coat assembly. What sort of evolutionary parsimony resulted in such widespread appearance of a single kind of protein module is not evident. Viruses can jump from plants to insects and from insects to vertebrates, so for these viruses, the recurrence of the jelly-roll β-barrel is unlikely to reflect a common origin that antedates host divergence. The recurrence is more likely the result of selection and gene exchange among already widely diverged host organisms.

The second recurring globular domain in viral coat proteins was discovered initially in the coat of dsDNA bacteriophage such as HK97 and subsequently found in most other dsDNA bacteriophages (T4, lambda, P22, etc.). This “HK97 fold” (Fig. 3.16) is also the core of the herpesvirus capsid subunit.[14,107,426] Like their bacteriophage cousins, herpesviruses pump their genome DNA into a preformed shell through a specialized icosahedral vertex and a dodecameric “portal protein.”[63,212,278,279] Adenoviruses, and probably their bacteriophage cousins like PRD1, with hexonlike capsid subunits, are also thought to insert DNA into a preassembled empty capsid, but the motors that effect the insertion seem to be

different from those in the herpesviruses,[189,287,433,434] and the coupling of packaging and DNA replication is different.[60] Thus, the structures of the coat proteins of two major classes of dsDNA viruses correlate with the machinery by which members of each of these classes package DNA.

Genome Packaging

Incorporation of viral nucleic acid must be specific, but it must also be independent of most of the base sequence of the genome. Therefore, viral genomes generally have a *packaging signal*—a short sequence or set of sequences that directs encapsidation. Recognition of the packaging signal depends on the nature of the genome and on the complexity of the assembly mechanism. For many plus-sense, ssRNA viruses, there is a direct interaction between the packaging signal and the capsid protein. dsDNA viruses insert genomic nucleic acid into a preformed shell, and genome recognition is a property of the virus-encoded packaging system. Negative-strand RNA viruses also rely on close coupling of replication and packaging of the RNA into an RNP.

Positive-Strand RNA Genomes

Viruses with plus-sense, ssRNA genomes (eg, most icosahedral plant viruses; picornaviruses; alphaviruses; flaviviruses) require no definite overall secondary or tertiary fold for the genomic RNA, aside from the restriction that it fits within the shell. This restriction is actually quite severe, and the RNA is packed very tightly, approximating the density of RNA in crystals.[271] Even random-sequence RNA contains a substantial fraction of its nucleotides in base-paired stem loops and related secondary structures[140]; to fit efficiently within the interior of a capsid, the RNA must pack with many of these segments of structured RNA in contact with the inward-facing surface of the shell. This result can be achieved by assembly around the RNA, without specific capsid-RNA interactions except for the packaging signal(s). Nonetheless, even in icosahedrally averaged crystal structures, segments of partially ordered RNA can often be detected, sometimes tucked into shallow grooves on the inner capsid surface.[72,120] Recent evidence, both for RNA bacteriophages and for enteroviruses, suggests that some of these segments resemble distributed packaging signals.[333,353]

In RNA bacteriophages such as MS2 and R17, a translational regulatory sequence that is probably also the principal packaging signal folds into a stem-loop structure (Fig. 3.21A).[20] A dimer of the coat protein, the assembly unit for the T = 3 particle, recognizes this structure and binds it specifically.[52,390,391] Bases in the loop and a looped-out base in the stem fit into a groove on the inward-facing surface of the subunit dimer; conserved bases make defined protein contacts. The interaction requires that the coat-protein dimer be in the A/B conformation (the conformation of the 60 dimers not centered on icosahedral twofold axes: Fig. 3.21B), and hence it is effectively a conformational switch, like the N-terminal arms in TBSV, SBMV, and many other T=3 plant viruses.[369] Degenerate versions of the specific packaging sequence are present throughout the genome; cross-linking studies and cryo-EM structures done without imposing icosahedral symmetry suggest that these more weakly binding elements may account for interactions with many of the A/B dimers, in addition to the dimer that binds the translational regulatory site.[91,211,333] The maturation protein, which replaces one dimer in the T = 3 lattice and which interacts with an F-pilus to deliver the RNA to a bacterial cell, binds stem loops at both ends of the genome.[91,211] The redundancy of packaging signal interactions can explain the robustness of specific assembly and its lack of sensitivity to mutations in the principal packaging signal. Cross-linking studies have suggested a similarly distributed set of degenerate packaging signals in parechoviruses—a group of picornaviruses.[353] Essentially all proposed or well-documented packaging signals have stem-loop structures, with one or more conserved nucleotides (or nucleotide types) exposed at the loop.

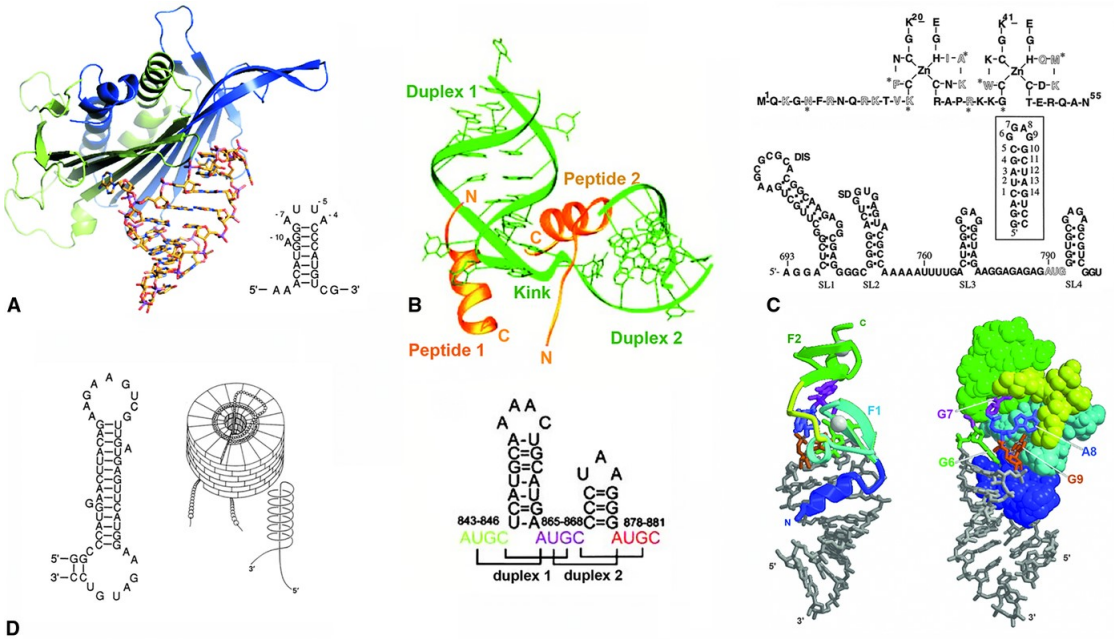

FIGURE 3.21 **Various modes of ssRNA recognition and packaging**. **A**. RNA bacteriophage. A stem-loop (sequence shown as *inset*) packs against the inward-facing surface of a protein-subunit dimer; there are specific contacts between residues in the protein and four unpaired bases (–4 –5, –7, –10). (Adapted from Valegård K, Murray JB, Stonehouse NJ, et al. The three-dimensional structures of two complexes between recombinant MS2 capsids and RNA operator fragments reveal sequence-specific protein-RNA interactions. *J Mol Biol*. 1997;270(5):724-738. Ref.[391]) **B**. Alfalfa mosaic virus. A folded, stem-loop, RNA structure (*green*) is a docking site for two N-terminal subunit arms (*gold*). The arms are unstructured until they associate with the RNA. (Modified from Guogas LM, Filman DJ, Hogle JM, et al. Cofolding organizes alfalfa mosaic virus RNA and coat protein for replication. *Science*. 2004;306(5704):2108-2111. Reprinted with permission from AAAS. Ref.[152]) **C**. HIV-1. Two "zinc-knuckle" domains (labeled F1 and F2 in the ribbon representation at

the *lower left*), near the C-terminal end of the Gag polyprotein (sequences shown at the *top*), bind a stem-loop structure in the packaging signal of the genomic RNA (*center and bottom*). Purine bases that have conserved stacking interactions are labeled in the surface representation at the *lower right*. Adapted from De Guzman RN, Wu ZR, Stalling CC, et al. Structure of the HIV-1 nucleocapsid protein bound to the SL3 psi-RNA recognition element. *Science*. 1998;279(5349):384-388. Reprinted with permission from AAAS. Ref.[96]) **D**. Tobacco mosaic virus.[47,387,444] The sequence at which RNA packaging initiates, shown on the left, is roughly 500 nucleotides from the 3′-end of the genome, and assembly of the helical particle proceeds by addition of subunits at one end of the growing helix, drawing the 5′ end up through the center of the particle, as shown on the right. Coating of the 3′ overhang proceeds more slowly at the other end of the particle.

Other nonenveloped, positive-stranded RNA viruses of eukaryotes recognize their genomic RNA, not through a groovelike site on the protein, but rather through a flexibly extended, positively charged protein arm, often at the N-terminus of the coat protein. There is an interaction of this kind between the coat protein of alfalfa mosaic virus (AMV) and a 3′-terminal segment of RNA.[152] A bound coat protein dimer is essential for replication—probably to recruit the RNA-dependent RNA polymerase. The same interaction is also likely to initiate packaging. The dimer contacts a pair of tandem RNA hairpins through a 26-residue, N-terminal arm. The two arms and the two RNA stem loops fold together into a well-defined structure, cross strutted by base pairing between conserved AUCG sequences that follow each stem (Fig. 3.21C). There are six such stem loops in the 3′ segment of the viral genome; it is possible that binding of three coat-protein dimers initiates shell assembly. Other stem-loop elements could in principle make weaker and less specific versions of this kind of interaction, but the net-positive charge of the N-terminal extensions may be sufficient for less specific condensation of the RNA.

The enveloped alphaviruses also have a multistem-loop packaging signal, recognized (with structural details not yet determined) by an extended, positively charged N-terminal arm of the nucleocapsid subunit.[39,40,126,206] There appears to some redundancy in the stem loops, all of which contain a GGG trinucleotide in the loop, as deletion of several of the eight stem loops does not compromise the efficiency of RNA packaging and virion assembly.

Retroviral packaging signals, known as *psi* sequences, associate specifically with the nucleocapsid (NC) domain of the Gag protein.[110] The dimeric 5′ leader of HIV-1 has over 20 such NC binding sites,[105] at least two of which associate specifically as stem-loop structures with two "zinc-knuckle" modules in HIV-1 NC[96,204,245] (Fig. 3.21D). The two zinc modules are flexibly linked in unbound NC, but they adopt a defined, three-dimensional organization in complex with the RNA. The association thus imparts additional order to the protein element and may also influence conformational equilibria in the RNA. Binding with lower affinity to more than the two psi-element stem loops for which structures have been determined may enhance specificity and facilitate RNA condensation,[105,245] like the lower-affinity packaging signals in picornaviruses and RNA bacteriophages. An RNA conformational switch may also accompany the initial event in packaging recognition.[105]

Assembly of helical structures requires unwinding of any base-paired stems in the RNA genome being packaged. TMV has evolved an assembly-driven helix-breaking feature into its *in vitro* characterized packaging pathway. Viral assembly begins at an internal origin sequence, about 1 kb from the 3′ end of the genome.[387,444] A 75-base sequence containing a presumptive stem-loop structure is sufficient to initiate specific encapsidation (Fig. 3.21E), which proceeds by a mechanism that requires that the 5′ end of the RNA be drawn through a channel along the axis of the assembling particle,[47] enforcing loss of local secondary structure and compensating for the free energy loss by favorable RNA-protein interactions at the growing end of the helix.

dsDNA Genomes

The best understood dsDNA packaging mechanisms are those of the tailed bacteriophages.[55,98,146,315] A pentameric ATPase, with a ringlike structure resembling that of a helicase, pumps DNA into the empty head, through a portal protein at a single, unique vertex, until it reaches a tightly coiled state, as illustrated in Figure 3.16E.[115,116,363] Removal of the scaffold leads in most cases to a substantial expansion of the head, accomplished through conformational rearrangements in the major capsid protein.[114,199] The head itself is either an isometric icosahedral shell (eg, lambda, P22, or HK97, Fig. 3.16) or a prolate one (eg, T4 or ϕ29: cf. Fig. 3.17A). In the latter cases, the scaffold protein directs the elongation. The ATPase dissociates from the portal after DNA packaging is complete,[146] allowing the portal protein to become the attachment point for the tail or DNA injection structure. The substantial internal pressure of the packaged DNA[363] may help drive injection into a target bacterium.

Various models have been proposed for the coupling of ATP hydrolysis with the concomitant transport of DNA into the head.[1,264,358,372-374] The last segment of DNA to enter remains in the channel of the portal protein. To avoid entanglement, it is likely that the leading end of the DNA attaches to the head interior.[160] In P22, a tubelike, inward projecting extension of the portal may also help direct coiling and prevent tangles[378]; closely related viruses lack the prominent tube, however. DNA insertion leads to formation of a tight, uniform coil (Fig. 3.16E). Because the DNA is tightly wound, the side to side spacing of adjacent segments is very regular; the value of this spacing is determined by the precise volume of the head and by the length of the inserted genome.[115] Viruses like bacteriophage lambda that replicate their DNA in a rolling circle mode couple DNA packaging with cleavage of the replicated concatemer. Others, such as ϕ29, have a virally encoded protein that primes synthesis of both DNA strands and that remains attached to the ends of the encapsidated genome.

DNA packaging into herpesvirus capsids closely resembles the process just described for the tailed bacteriophages (see Chapter 9, volume 2). Not only does the shell-forming domain of the major capsid protein have the HK97 fold,[14] as described above, but the portal protein, attached to a unique vertex, likewise resembles its tailed-phage counterpart.[189,241,279,327] As in dsDNA bacteriophages, the tightly packaged DNA is a uniformly spaced, liquid crystalline–like coil (Fig. 3.20), and a particularly well-ordered coil of DNA wraps around the inward-projecting surface of the portal protein.[241] The wrapping may be a sensor for DNA pressure within the head, creating a conformational signal for the transition from packaging to subsequent maturation steps (eg, concatemer cleavage by terminase, tail attachment for a bacteriophage). An alternative proposal, which does not exclude the head-full signaling function, is that it represents the attachment point for the end of the genome that enters first.

Adenoviruses have, near the left-hand end of their linear genome, a set of AT-rich repeats that determine DNA incorporation into virions (see Chapter 4, volume 2). Virions contain about 1000 copies of a protein (VII) with strong positive charge, and it is believed that this protein condenses the viral DNA within the virion core and that it may remain associated with the DNA after uncoating. In the adenovirus-like

bacteriophage PRD1, there is a unique vertex defined by the presence of proteins required for DNA packaging and injection.[139] One of these, the ATPase, is distantly related to protein IVa2 of adenoviruses and more closely related to candidate packaging ATPases for other dsDNA viruses with internal membranes.[189] Adenovirus mutant lacking IVa2 assemble only empty capsids. A separate adenovirus portal protein has yet to be identified.[5]

Polyoma- and papillomaviruses incorporate cellular histones, so that the closed, circular DNA comprises about 20-25 nucleosomes (see Chapters 1 and 2, volume 2, for references). This minichromosome is further condensed as the capsid assembles around it. Packaging of the minichromosome of polyomaviruses appears to be directed by sequences in a histone-free region.

dsRNA Genomes

The segmented genomes of dsRNA viruses require packaging mechanisms that ensure, like those of the negative-strand ssRNA viruses with segmented genomes, incorporation of a complete genomic complement. The packaged RNA is a capped, plus-sense strand; the incorporated pRdRp produces the complementary strand within the assembled particle.

Complementary studies of reo-, rota- and orbivirus assembly suggest that eukaryotic dsRNA virus genome packaging begins with association of one each of the various plus-strand RNA segments into a single complex, held together largely by specific RNA-RNA interactions.[31] Evidence for initial formation of such a "complete genome" comes from successful *in vitro* assembly of bluetongue virus (BTV) cores, which requires all 10 plus-strand segments, as well as the coincorporated RdRp and the capping enzyme.[244] For rotaviruses, the nonstructural protein 2 (NSP2), with high affinity for single-strand RNA, chaperones this process by destabilizing intrasegment secondary structure and facilitating formation of specific intersegment interactions.[32,36,179,192] The reovirus σNS protein may have a similar role.[30,428] Rotavirus NSP2 is an octamer with a central channel that could accommodate ssRNA.[192,197,217,381] A second nonstructural protein, NSP5, appears to compete with RNA for binding to NSP2, suggesting it may have some sort of cochaperone-like activity.[197] Sequences covering untranslated regions at both 5′ and 3′ ends of the segments probably contain the relevant recognition functions.

The rotavirus RdRp recognizes the 3′ end of the plus-strand though interactions in the template entry channel, likely as part of the specific packaging mechanism.[247] Other eukaryotic dsRNA virus RdRps could have similar specificities, although those have not yet been shown. Binding the 5′ cap at the cap-binding site (present on both reovirus and rotavirus RdRps[247,379]) may make the overall association tighter. For reo-, rota-, and orbiviruses, one RdRp binds specifically with a shell-protein decamer (Fig. 3.12). Thus, the RdRps associated with the 3′ ends of the genome segments in the multisegment complex, each also associated with a shell-protein, could coordinate coassembly of the shell with the RNA complex,[31] completed by addition of any required RNA-free shell-protein decamers to make a full, 12-decamer complement. Generation of the complementary strand of each segment inside the assembled particles results in a tight, liquid-crystalline packing or dsRNA, like the head-full spool of dsDNA in bacteriophages. The end of each dsRNA segment remains associated with the RdRp, but in reoviruses visualized by cryo-EM reconstructions, the rest of the segments extend away from the polymerase, rather than spooling around it.[184,235,294,436]

The various eukaryotic dsRNA viruses have between 9 and 12 genome segments. Each of the corresponding 9-12 RdRps is fixed within the core-shell close to a fivefold axis, the location at which a transcript leaves a transcribing particle[195,230] (Fig. 3.12). To enable reinitiation for the many rounds of transcription that occur when the inner capsid particle is released into the cytoplasm of an infected cell,[379] each polymerase remains associated with the 3′ end of the template (noncoding) strand of its corresponding genome segment, by binding the 5′-cap of its complementary nontemplate strand at a site near the template entry channel.[247,379] Otherwise, that terminus would "get lost" in the particle interior, greatly delaying finding an unoccupied polymerase. The likely mechanisms for some of these steps have been worked out for rotaviruses, orthoreoviruses, and orbiviruses.

Packaging of bacterial dsRNA viral genomes proceeds by a quite different mechanism from the one just outlined for their eukaryotic counterparts.[261,311] A procapsid assembles, into which each of the three positive-stranded RNA segments inserts sequentially, in a specific order. Minus-strand synthesis then occurs inside the shell, as in the eukaryotic viruses. The procapsid includes the major shell protein (similar to that of reovirus or rotavirus), the polymerase, an ATPase, and a protein thought to serve as an assembly "clamp." One copy of the hexameric ATPase, which may be a packaging helicase for plus-sense RNA, analogous in function to the packaging proteins of dsDNA bacteriophage, lies at each fivefold vertex[97]; RNA insertion appears to occur at only one of these twelve positions, however, even though all are occupied by an ATPase.

Negative-Strand RNA Genomes

The nucleocapsid proteins (N) of negative-strand RNA viruses with single-segment genomes (eg, the rhabdoviruses, VSV and rabies virus, and borna disease virus) all have closely related structures.[6,142,340] Recombinant N proteins from VSV and rabies viruses bind nonspecific RNA from the expression host and form rings of 10-14 subunits, with an N-terminal arm that embraces one neighbor in the ring and a loop near the C-terminus that extends into the other neighbor. The subunits have two lobes with a groove between them that faces the center of the ring and binds the RNA—9 bases per subunit. The recombinant VSV-N ring, a more tightly wound and circularized version of the helical ramp that the nucleocapsid forms in the bullet-shaped virion (Fig. 3.22), approximates the initial turn at the domed end of the particle, which has the 3′ end of the RNA. For transcription or replication, the polymerase must withdraw the RNA from the groove in the RNP.[6,142] The interactions of neighboring N proteins, which embrace each other through N-terminal arms and C-proximal loops, probably keep the chain of N-proteins intact even when the RNA separates from it to enter the polymerase.[193,231] The polymerases and their cofactors also organize association with the nucleocapsid protein as the nascent genome emerges from the enzyme, avoiding formation of any secondary structure and protecting the nascent RNA from recognition by innate-immune receptors in the cell.[176,193,231]

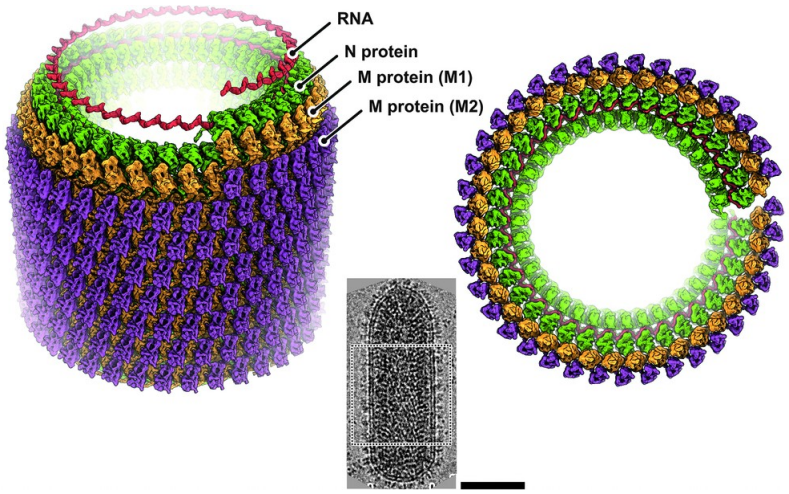

FIGURE 3.22 **Organization of RNP and M in vesicular stomatitis virus (VSV)**. *Inset* shows a cryo-EM image of a VSV particle. "Spikes" of the trimeric glycoprotein (G) project from the lipid bilayer membrane. Scale bar: 50 nm. The uniform shaft of the bullet-shaped particle is a regular helical assembly of the RNP with the M protein. There are two (identical) M subunits for each N subunits, although the outer (M2) layer is not fully occupied. The figure shows a side view and an axial view of the RNP-M1-M2 assembly. A flexible, N-terminal extension of the M subunit (not shown) interacts with the inner leaflet of the membrane bilayer; this interaction and coassembly of M and RNP appear to drive budding of the particle. (Adapted from Jenni S, Horwitz JA, Bloyet LM, et al. Visualizing molecular interactions that determine assembly of a bullet-shaped vesicular stomatitis virus particle. *Nat Commun*. 2022;13(1):4802. http://creativecommons.org/licenses/by/4.0/. Ref.[94])

Orthomyxoviruses have six to eight genome segments, packaged as ribonucleoprotein complexes (RNPs), which resemble rods folded back on themselves and coiled in a double-helical arrangement, with the three-subunit polymerase bound at one end and a loop at the other[9,117,263] (Fig. 3.23E). The lengths of the eight RNP rods in influenza A, between 300 and 1200 Å, correspond to the eight genome segment lengths, when coiled as just described; their diameter is about 120 Å.[284] Complementary sequences in the 5′ and 3′ noncoding regions determine the folded-back arrangement and bind the polymerase; sequences at either end of the coding region also contribute to specific packaging.[138,272] Serial transverse sections through elongated buds of influenza A show eight rods, seven around one, about 120 Å in diameter, extending for variable distances from the tip of the bud toward its cell-proximal end; the distances correspond to the lengths of the various genome segments.[274,284,285] Tomographic reconstructions of purified filamentous influenza virus particles show that this internal organization is retained in the budded particle (Fig. 3.23E).[50] Interfering with packaging of one segment reduces packaging of others.[272] Thus, there appears to be a sequential recognition mechanism to ensure a proper complement of genome segments, perhaps formally (although not structurally) analogous to the selection mechanism in dsRNA viruses. The influenza NP-protein, like those. Ref.[117] of the single-segment negative-strand RNA viruses, has two lobes with an RNA-binding groove between them, but the folded structures are not identical.[419] The groove that accepts RNA faces outward in the double-helical RNP and looped-out RNA could dictate specific incorporation of the eight segments.[117] The RNA that forms the central element in the seven around one arrangement may have a particularly critical role; some evidence suggests that this segment is the one that encodes polymerase subunit PB2.[272] Influenza C and D, which have only seven genome segments, still show a 7+1 arrangement, indicating a conserved packaging mechanism; the eighth segment might be a host-cell RNA or a redundant copy of one of the seven viral RNAs.[273]

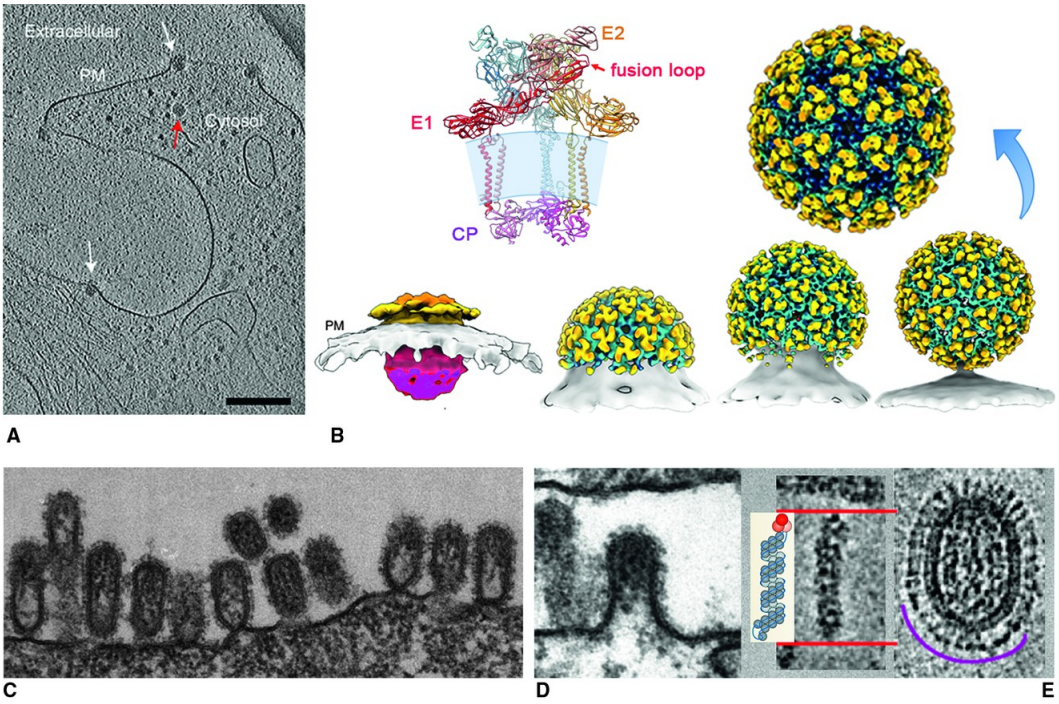

FIGURE 3.23 **Budding of enveloped viruses**. **A, B**. Alphaviruses: chikungunya virus budding at the plasma membrane. (Adapted by permission from Nature: Chmielewski D, Schmid MF, Simmons G, et al. Chikungunya virus assembly and budding visualized in situ using cryogenic electron tomography. *Nat Microbiol*. 2022;7(8):1270-1279. Ref.[74]) **A**. Cryo-ET of viruses budding at the thin edge of an infected cell. Nucleocapsids assemble in the cytosol (*red arrow*). At the plasma membrane, they recruit glycoproteins E1 and E2 (*white arrows*), which are synthesized on endoplasmic reticulum–associated ribosomes and transported through the secretory pathway to the cell surface. Scale bar: 200 nm. **B**. Molecular events in budding. *Upper left*: An E1-E2 heterohexamer (E1 in *red*; E2 in *orange*), recruited by three core protein subunits, through contacts with the E2 cytoplasmic tail. The one-to-one interaction ensures coherence of the T = 4 icosahedral lattices of the core and the external glycoprotein array. Semitransparent *blue arc* represents the lipid bilayer of the virion. In the prefusion structure at neutral pH, one domain of E2 covers the fusion loop on E1. At the acidic pH of an endosome, conformational changes in the glycoproteins allow E1 to project outward and the fusion loop to insert into the endosomal membrane. The representation of an assembled virion (*upper right*) illustrates the arrangement of the 80 E1-E2 heterohexamers on the surface of a virus particle. *Lower row*: Steps in budding, culminating in pinching off (*curved arrow*) of the infectious virion (*upper right*). Nucleocapsid: *magenta*. Surface representation of glycoproteins E1 and E2, in colors from *yellow* (outermost) to *dark green* (membrane proximal), according to distance from the center of the particle. **C**. Influenza virus. Thin section electron microscopy of slightly elongated virions budding from the surface of an infected cell. The longer particles show that the eight RNPs cluster at the leading tip, as illustrated by the budding particle in **(D)**. (Adapted by permission from Nature: Noda T, Sagara H, Yen A, et al. Architecture of ribonucleoprotein complexes in influenza A virus particles. *Nature*. 2006;439(7075):490-492. Copyright © 2006 Springer Nature. Ref.[284]) **E**. Slice through a cryo-tomogram of a complete virion, showing the HA cluster of RNPs, the array of HA trimers, and the more limited region at the trailing tip (the last to bud) of NA. (Adapted with permission from Calder LJ, Wasilewski S, Berriman JA, et al. Structural organization of a filamentous influenza A virus. *Proc Natl Acad Sci U S A*. 2010;107(23):10685-10690. Ref.[50]) An isolated RNP is shown at roughly the same magnification on the *left*,[50] together with a schematic diagram of its helical-hairpin organization (Isolated RNP adapted by permission from Nature: Eisfeld AJ, Neumann G, Kawaoka Y. At the centre: influenza A virus ribonucleoproteins. *Nat Rev Microbiol*. 2015;13(1):28-41. Copyright © 2014 Springer Nature. Ref.[117]). The three-subunit RNA-dependent RNA polymerase (*red*) associates with the RNA termini at the membrane-proximal end. The headgroup layer of the inner leaflet of the membrane bilayer appears denser than the headgroup layer of the outer leaflet, because of its close association with M.

Viral Membranes

Most enveloped viruses (the large and very complex poxviruses and the membrane-containing bacteriophages are exceptions) acquire a lipid bilayer and associated viral transmembrane proteins by budding through a membrane of the host cell—either the plasma membrane (eg, alphaviruses, orthomyxo- and paramyxoviruses, rhabdoviruses, filoviruses, retroviruses), the ER (flaviviruses), the ER-Golgi intermediate compartment (coronaviruses), the Golgi (bunyaviruses), or the inner nuclear membrane (herpesviruses) (Fig. 3.23). The viruses direct insertion of their surface glycoproteins into the relevant host-cell membrane, through standard cellular compartmentalization pathways, such as transport through the trans-Golgi network to the cell surface or retention in the ER or Golgi; the driving force for budding may then come from membrane-coupled assembly of the internal structures, as in assembly of rhabdoviruses,[194,442] orthomyxoviruses,[305,306] and retroviruses,[375] from lateral interactions in the glycoprotein layer, as in budding of flaviviruses and of at least some bunyaviruses,[182,371,396] or from some combination of the two, as in budding of alphaviruses[41,48] (Fig. 3.23A).

The mechanisms for specific capture of the viral glycoprotein(s) by the internal components—only roughly characterized, if at all, in many cases—vary widely. The simplest mechanism, a one to one interaction of the surface glycoprotein with a membrane-facing internal protein, appears to be the case only for alphaviruses,[288,432,440] which have a T = 4 icosahedral organization for both the glycoprotein surface layer and the internal nucleocapsid (core) particle[21,22,70,73,215,270,310,316,393,395,432,435] (Fig. 3.23B). The icosahedrally organized flavivirus particles (Fig. 3.12)[73,215,280,310,314,351,359,393,395,437,439] have much weaker interactions between envelope and core proteins,[384] and expression of just the viral glycoproteins (prM and E) is sufficient to yield budded, viruslike particles with surface structures similar to those of intact virions.[7,259]

Specificity of membrane-protein incorporation is not always absolute. For example, HIV-1 buds with only a few copies of its glycoprotein on the virion surface (Fig. 3.24), and some host-cell membrane proteins can incorporate into the particle.[45,154] In the well-studied example of VSV, ectopic expression of a heterologous viral glycoprotein leads to phenotyptic mixing—incorporation of the heterologous protein into the progeny particles.[258,443] VSV chimeras, in which the coding region for a heterologous glycoprotein replaces that for VSV G thereby avoiding any mixed incorporation,[51,133,347,392] and lentivirus chimeras, with related substitutions,[198] also propagate successfully. VSV and lentivirus chimeras are particularly useful for studying entry properties of human pathogens without requiring high-level containment.

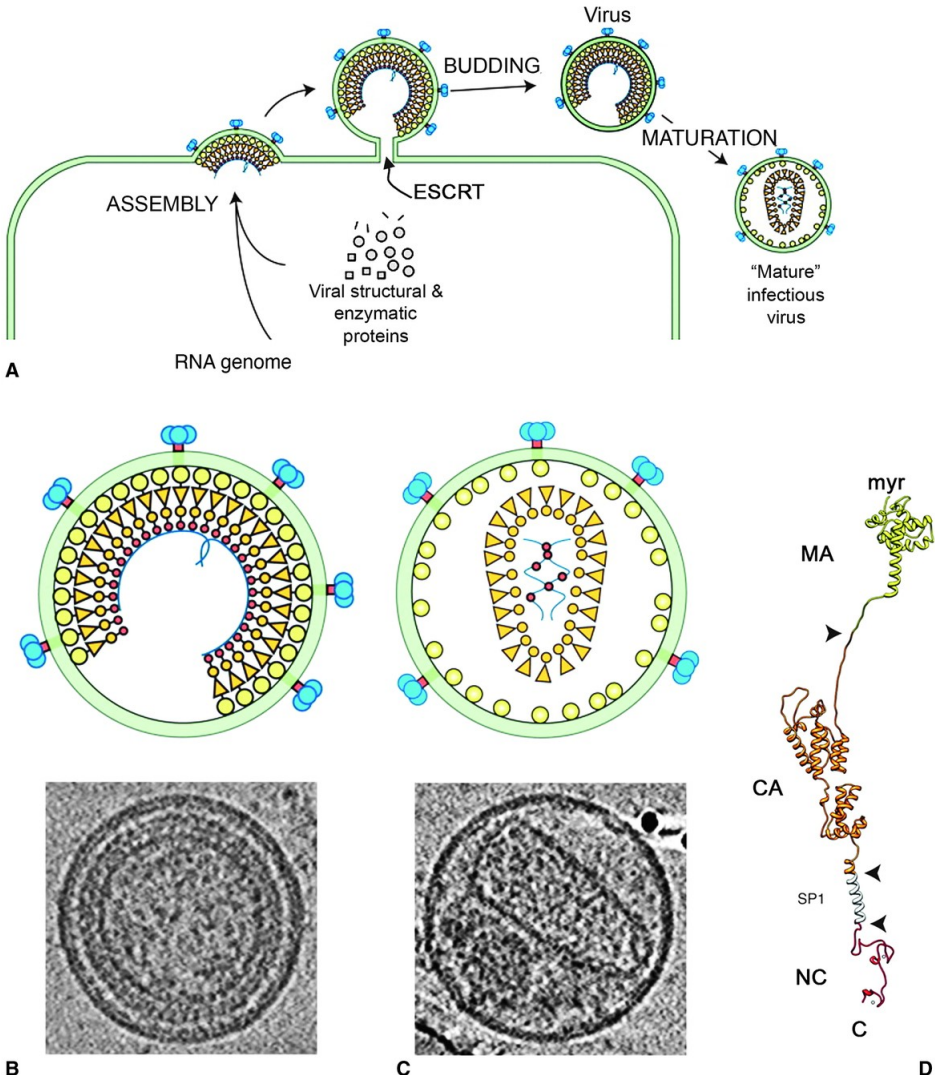

FIGURE 3.24 **Cleavage and rearrangement of HIV-1 Gag**. **A**. HIV budding at the cell surface. Pinching off requires the ESCRT pathway. **B**. Immature particle, the immediate product of budding. The Gag domains are MA (*yellow*), which interacts with the membrane both through a myristoylated N-terminus and a positively charged, membrane facing surface, CA (*orange*), which has two folded modules, and NC (*red*), which interacts with the genomic RNA (see Fig. 3.21C). **C**. Mature particle. Cleavage of Gag, by the viral protease (a domain of the Gag-Pol fusion protein produced by ribosomal frameshifting), leads to rearrangement of CA into a fullerene cone (see Fig. 3.17B) and condensation of the genomic RNA within it. *Upper panels* are schematics; *lower panels* are slices through cryo-tomograms. **D**. Cartoon of Gag, with protease cleavage sites shown as arrowheads. SP1 is a spacer peptide. (Adapted with permission from Sundquist WI, Krausslich HG. HIV-1 assembly, budding, and maturation. *Cold Spring Harb Perspect Med*. 2012;2:a006924. Copyright © 2012 Cold Spring Harbor Laboratory Press. Ref.[375])

Except for alphaviruses, flaviviruses, and members of the one family of bunyaviruses,[182,396] the sizes and shapes of most enveloped viruses vary to some degree from particle to particle. Budding of many of these pleomorphic particles depends on a "matrix" (M) protein, which lines the inner face of the viral membrane. Although the subunit structures of M proteins from different groups of viruses vary, they have analogous functions in driving bud formation and in recruiting the nucleocapsid.[385] Most are not integral membrane proteins, although coronavirus M, with a short ectodomain, three transmembrane helices, and a cytoplasmic domain of about 100 residues, is an exception.[99,275] Filamentous influenza virus particles provide an extreme example of the role of a matrix protein in budding.[282,283] Even very long filaments still contain only one eight-segment genome complement at their leading end.[75,284,285] The helical organization of the influenza-virus matrix protein, M1, allows budding to continue to arbitrary lengths under circumstances that drive it to do so[229,305,306] (Fig. 3.25). The VSV matrix protein (M) coassembles with the nucleocapsid into the helical shaft of the bullet-shaped virion; in that case, the length of the particle is fixed by the length of the RNA (Fig. 3.22). The absence of a matrix protein in bunyaviruses[183]—the largest group of enveloped viruses—appears to require that the glycoprotein (GPC, cleaved to Gn and Gc) alone provide the driving force for budding. The cytoplasmic "tail" of Gn directs RNP incorporation, and some evidence suggests that it has a matrixlike function.[371]

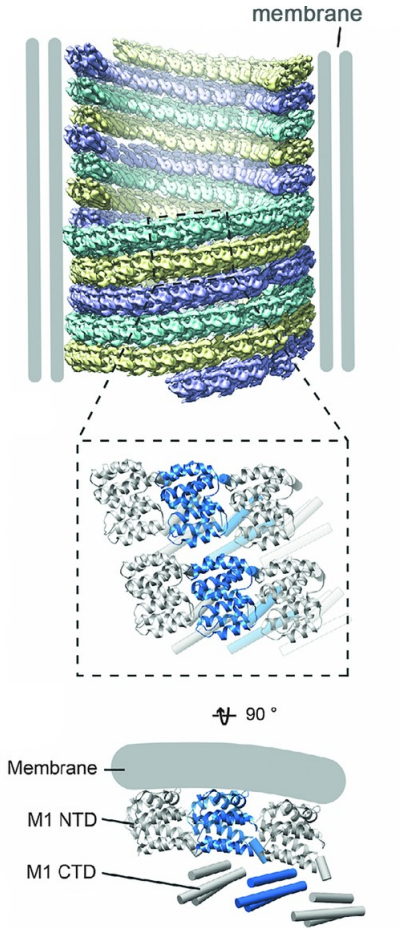

FIGURE 3.25 **Influenza M1 protein helical array beneath the membrane of a filamentous influenza H3N2 virus particle, imaged by cryo-ET with subtomogram averaging (EMD-11075, PDB-6Z5J)**.[306] *Top*: The two-domain M1 subunit assembles as a three-start helix (each in a different color). *Center*: Magnification of two adjacent helical turns, with alternate subunits in *white* and *blue*. *Bottom*: Axial view of one turn. The C-terminal domain (CTD) is not as well ordered as the N-terminal domain (NTD), and the figure shows only the CTD alpha helices, as cylinders. (Adapted from Peukes J, Xiong X, Briggs JAG. New structural insights into the multifunctional influenza A matrix protein 1. *FEBS Lett*. 2021;595(20):2535-2543. http://creativecommons.org/licenses/by/4.0/. Ref.[305])

Pinching off from the cell surface, or into the lumen of the ER or Golgi, does not require a cellular pinching activity for alphaviruses and flaviviruses; membrane-linked assembly of viral components probably provides the force needed to distort and sever the membrane bilayer.[382,405] In many other cases, however, completion of budding requires that the virus recruit components of a cellular budding machinery.[312] In the examples of such recruitment that have been studied in some detail (particularly HIV and other retroviruses, but also rhabdoviruses, filoviruses, arenaviruses, and some paramyxoviruses[406]), the virus redirects to the cell surface a set of protein complexes (the ESCRT machinery) that normally function at late endosomes, where they generate pinched-off invaginations into the endosomal lumen to create multivesicular bodies (MVBs).[134,370,398] The topology of viral budding from the cell surface is the same as the topology of lumenal vesicle formation (away from the cytosol), and the ESCRT mechanism that releases the virus particle is presumably the same as the mechanism that generates the vesicular content of MVBs. A motif in Gag or M, known as a "late domain," recruits specific ESCRT components, which then initiate assembly of the rest of the elaborate ESCRT complex.

Budding of influenza virus is ESCRT independent[66]; instead, an amphipathic helix in M2, the small, tetrameric proton channel that allows the interior of the particle to acidify before release from an endosome, appears to potentiate scission.[335] Selective incorporation of NA at the base of the budding virion[50] could in principle also be part of the scission mechanism. Although association of HA and NA with lipids characteristic of cholesterol-rich microdomains[346,377,429] has been proposed to contribute, there is no clearly defined mechanism for how lipid compositional discontinuities might lead to membrane scission.

The lipids in the viral membrane derive from the cell, and to a first approximation, the incorporated lipids represent a sample of those in the membrane through which the virus budded.[209] Viruses that emerge through the plasma membrane contain phospholipid and cholesterol in characteristic proportions, whereas those that emerge into the lumen of the ER contain almost no cholesterol. Cholesterol tends to increase the thickness of a bilayer, by restricting rotation about single bonds in the fatty-acid chains of adjacent phospholipids.[317] The lengths of α-helical transmembrane segments in viral glycoproteins vary accordingly: from about 26 residues in the HA of influenza virus (which buds at the cell surface) to 18-20 in the E protein of yellow fever virus (which buds into the ER). Within these overall regularities, specific examples show that viral envelope proteins can specify selective glycerophospholipid incorporation. For example, glycerophospholipid profiles of three different influenza virus strains grown in eggs have a substantially higher phosphatidylethanolamine (PE) content than do cells in noninfected allantoic fluid, and their compositions also differ slightly from one strain to another[185]; human cytomegalovirus particles have a similarly enhanced PE content.[239]

Postassembly Steps

For most viruses, and nearly all viruses that infect animal cells, one or more irreversible steps intervene in forming the mature, infections virion—very often, those steps include a proteolytic cleavage. The picornaviruses, already described, are a good example. In an infected cell, the principal structural proteins are cleaved from a polyprotein precursor (by a viral protease) before particle assembly, but one final, autocatalytic cleavage step occurs a Ref.[375] fter assembly—the scission of a peptide bond between VP4 and VP2 (see Chapter 2, volume 1 and caption to Fig. 3.8). The cleavage depends on the three-dimensional arrangement of the scissile bond, as found in a newly assembled precursor particle. Rearrangements of parts of the subunits following the cleavage stabilize the now mature, infectious virion. Even if processing of a precursor polyprotein is not involved, cellular or extracellular host proteases mediate critical steps in the maturation of many viruses. Many of the surface glycoproteins that facilitate membrane fusion during entry of enveloped viruses require proteolytic activation (see section on membrane fusion)—either by a furinlike protease late in the secretory pathway, by an extracellular protease after release of the assembled particle, or by a protease in the new host cell.[162]

Specific, postassembly proteolytic cleavage usually has two consequences. First, as in picornaviruses or many viral fusion proteins, it leads to a local rearrangement of polypeptide chains that stabilizes the structure. During retrovirus maturation, reorganization of the internal structure, required for infection, depends on cleavage of the Gag polyprotein by the viral protease present inside the budded particle (Fig. 3.24). Second, it allows the structure to undergo a much larger reorganization when "triggered" by binding of a specific ligand. Thus, when a mature poliovirus particle binds its receptor, an expansion occurs that allows VP4 to escape and to interact with adjacent membrane—a critical first step for translocating the particle or its genome from an endocytic compartment into the cytosol.[35,153,268] Likewise, fusion proteins of enveloped viruses undergo large-scale, fusion-promoting conformational changes when suitably triggered—for example, by binding protons in acidic endosomes or by binding a receptor or coreceptor—but in most cases, only if a critical cleavage has occurred.[161,162,360] The cleaved structure is "metastable" (but *not* the uncleaved precursor—a common misunderstanding): a high kinetic barrier separates it from the true energy minimum, in a conformation that would have been inaccessible before the cleavage. The barrier can be so large that the virus remains infectious at 4 °C for many weeks or months. Ligand binding (receptor; protons; etc.) lowers the kinetic barrier, leading to a rapid conformational rearrangement, coupled in most cases to an important step in viral entry.

Structural Biology of Virus Entry

Receptors and Coreceptors

There are no simple generalizations about virus receptors and how they bind with viral surfaces (Fig. 3.26). Viruses are "opportunistic": they evolve to recognize cell-surface molecules—for example, carbohydrates on glycolipids or glycoproteins (Fig. 3.26A) or proteins (Fig. 3.26B)—on cells of different tissues or different hosts, and they change or widen their tropism as they adapt to new surface molecules. For example, influenza virus recognizes a sialic acid-containing carbohydrate for cell attachment[301] (Fig. 3.26C). One or two mutations can change its preference from the predominant sialic acid linkage on avian cells (α-2,3) to the predominant upper-airway cell linkage in humans (α-2,6)—an important requirement for influenza viruses of avian origin to acquire human-human transmissibility (see Refs.[130,362] and Chapter 14, volume 1, for references). Most viruses have also evolved a mechanism to avoid "getting stuck" at the cell surface when emerging from an infected cell, as illustrated by the receptor-destroying activities of influenza viruses.[130] Many viruses simply bind weakly to their receptors and thus can dissociate in a reasonable time. The virulence of polyomavirus in mice is inversely related to viral affinity for its sialoglycoconjugate receptor (Fig. 3.26A), demonstrating that spread in the animal host, rather than entry into cells, is the principal correlate of pathogenesis.[23] HIV-1 has several mechanisms for downregulating its receptor (CD4) after infection, both to avoid envelope-receptor interactions within the secretory pathway and to facilitate viral release after budding (see Chapter 17, volume 3).

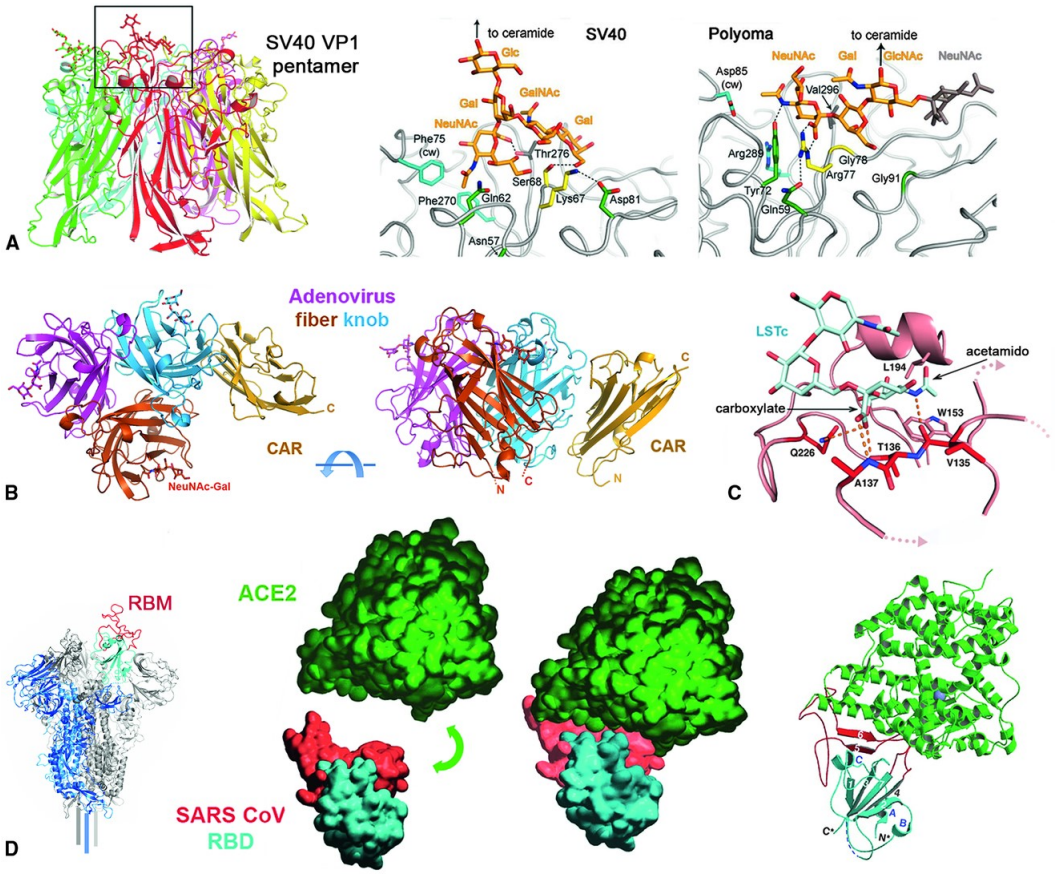

FIGURE 3.26 **Examples of virus-receptor interactions**. **A**. SV40 and polyomavirus. *Left*: Pentamer of SV40 VP1, showing location of the interaction on the outward-facing surface of the subunit. For reference to full virion, see Figure 3.14. *Right*: Detailed views of the receptor binding sites for the two viruses (*boxed region* in the left-hand panel), showing interactions of distinct glycan structures (from glycolipid headgroups) with homologous sites on the VP1 subunits. (Adapted with permission from Neu U, Woellner K, Gauglitz G, et al. Structural basis of GM1 ganglioside recognition by simian virus 40. Proc Natl Acad Sci U S A. 2008;105(13):5219-5224. Ref.[277]) **B**. Adenovirus penton-fiber knob, bound with domain 1 of the multi-Ig domain receptor, coxsackievirus-adenovirus receptor (CAR) and with sialic acid, which helps recruit type 2 adenovirus to cell surfaces. (Adapted with permission from Whittle JR, Zhang R, Khurana S, et al. Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. *Proc Natl Acad Sci U S A*. 2011;108(34):14216-14221. Refs.[349,407]) **C**. Influenza virus: sialic-acid binding site on the HA1 "head." **D**. SARS coronavirus (SARS-CoV): docking of the ACE2 receptor with the receptor-binding domain (RBD) of the viral glycoprotein spike.[226] The homologous interaction is essentially identical for the SARS-CoV-2 glycoprotein. *Left*: Cartoon representation of the spike glycoprotein ectodomain, with one RBD in an "up" position, and the other two in "down" positions[427] (PDB 5X5B). The subunit with RBD up is in color; the other two in *gray*. The up RBD is in *cyan*, and the receptor binding motif (RBM) on its outward-facing surface in *red*. ACE2 requires the up configuration to access the RBM. An α-helical, coiled-coil stalk, represented schematically, connects the structure shown with the transmembrane anchor of the glycoprotein. *Center*: ACE2 docking. Surface representations of ACE2 (*dark green*) and a single RBD (*cyan and red*). *Right*: The docked receptor in cartoon representation. (Adapted from Li F, Li W, Farzan M, et al. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science*. 2005;309(5742):1864-1868. Reprinted with permission from AAAS.)

Many viruses require a cascade of at least two distinct receptors—one for initial cell attachment and a second for triggering fusion or penetration. The receptor used for initial attachment may be a widely distributed molecule, like sialic acid or other glycans—for example, heparan sulfate for HSV-1—or it may be a quite specific protein, like the adenovirus receptor, CAR (Fig. 3.26B), or the HIV-1 receptor, CD4 (Fig. 3.27). The molecule that triggers fusion or penetration is sometimes called a "coreceptor"—for example, the chemokine receptors for HIV-1. In the case of HIV-1, CD4 primes the envelope glycoprotein to bind the coreceptor, which in turn induces fusion activation (Fig. 3.27). An obligate order of this sort will also obtain when the primary receptor is on the cell surface and the coreceptor is on the lumenal surface of an endocytic compartment.

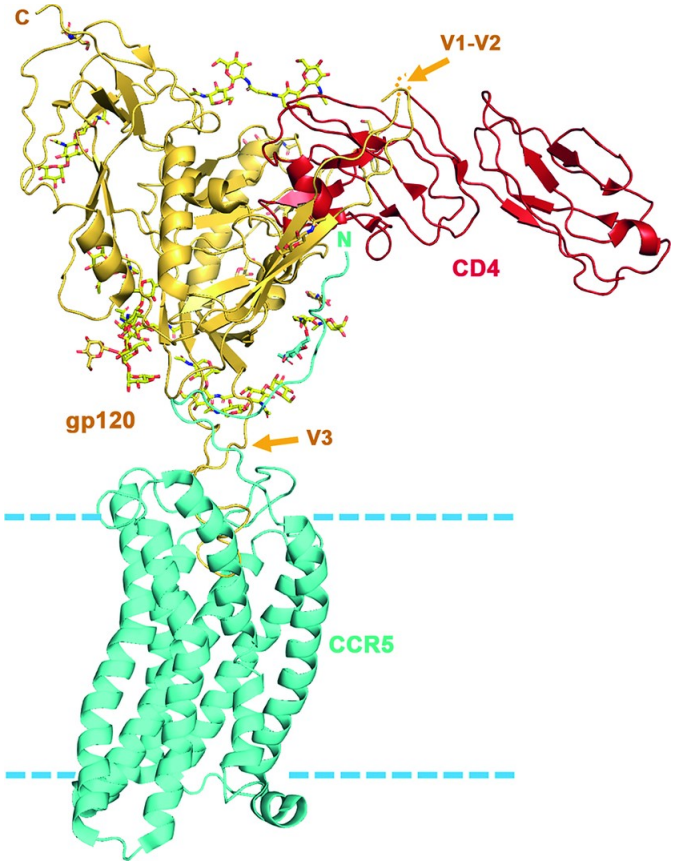

FIGURE 3.27 **HIV-1 gp120 interaction with receptor and coreceptor, illustrating how receptor (CD4) binding stabilizes conformational changes that allow coreceptor (CCR5) binding**. The first two (of four) domains of CD4 are in *red*; the receptor- and coreceptor-binding domain, gp120, of HIV-1 Env is in *yellow*; the coreceptor, CCR5, in *cyan*. CD4 binding displaces the V3 variable loop (labeled) from its position in unliganded Env trimer (in which it tucks under the V1-V2 variable loop, also labeled, now instead in contact with the first domain of CD4); the displaced V3 inserts deep into the ligand-binding pocket of the CCR5 chemokine receptor, while the long N-terminal, extracellular arm of CCR5 binds along base of the V1-V2 loop. (Figure made from PDB 6MEO . Shaik MM, Peng H, Lu J, et al. Structural basis of coreceptor recognition by HIV-1 envelope spike. *Nature*. 2019;565:318-323. Ref.[352])

Viral mutations can lead to amino acid residue changes that reduce recognition by the immune system (eg, influenza, HIV), and viral surface proteins have generally evolved structural features to sequester their conserved, receptor-binding surface from interaction with antibodies and potentially variable surfaces elsewhere. The footprint of an antibody combining site is substantial, so that even if a receptor site is exposed, it rarely matches the full extent of the surface within which amino acid residue changes will lower antibody affinity and hence escape neutralization.[79] Some viral receptor sites (eg, those on certain picornaviruses—Fig. 3.4) lie within a "canyon"[336,338]—a groove or pocket too narrow to admit the antigen-combining end of an antibody. Antibodies with unusually long or prominent heavy-chain CDR3 loops can often penetrate relatively tight cavities, but the periphery of the receptor site can generally mutate in ways that interfere with antibody binding but leave critical receptor contacts unchanged. The receptor-binding proteins of viruses such as HIV-1 (Fig. 3.27) and SARS-CoV (Fig. 3.26D) require a conformational change to expose the binding site completely. The receptor-binding motifs (RBMs) on the SARS-CoV and other coronavirus spike proteins are unusually extensive,[226] and they can encompass nearly the entire footprints of some antibodies (Fig. 3.26D). A conformational change, from receptor-binding domain (RBD) "down" to RBD "up," exposes the RBM; capture by the receptor prevents return of the RBD to its "down" conformation as long as the receptor remains bound.[416,427] The conformational dynamics thus minimize exposure of a critical epitopic region recognized by neutralizing antibodies. Moreover, because certain residues critical for antibody binding are less critical for receptor binding, immune escape mutations within the RBM do occur, as illustrated by the succession of variants during the SARS-CoV-2 pandemic.

The receptor or coreceptor interaction can have viral-entry functions other than attachment or endocytosis. For example, when poliovirus and other picornaviruses bind receptor, the interaction triggers expansion of the viral shell. Exit of VP4 from the expanded particle not only makes the rearrangement irreversible[24,153] but also allows VP4 to insert into the target-cell membrane, where it appears to help create an RNA transfer channel (see below). The receptor is a catalyst that lowers the energy barrier to an irreversible reorganization.[386] The function of this reorganization is viral entry; the triggering mechanism has evolved to allow the reorganization to occur only in an appropriate location. Reoviruses (Fig. 3.18) have an outer-layer protein, σ3, that caps the penetration protein, μ1.[109,233] Proteolytic removal of σ3 is required to make the particle competent to attach and penetrate (see Chapter 11, volume 3). There is, in addition, an essential autolytic cleavage of μ1,[281] with release of a myristoylated N-terminal peptide (μ1N) that forms pores in membranes. As described below, influenza HA folds in the ER into a stable, trimeric structure. Cleavage of one peptide bond in the HA_0 polypeptide chain primes the protein to undergo the low pH-triggered rearrangement that mediates fusion of viral and target-cell membranes. Although the barrier to rearrangement is so great at neutral pH that no conformational change occurs, proton binding in the low pH environment of the endosome lowers this barrier and triggers refolding. For HA, protons have a role analogous to that of some enveloped viral coreceptors, such as the chemokine receptors for HIV-1 or Niemann-Pick C1 for Ebola virus.[51]

Bilayer Fusion

The bilayer fusion reaction common to all the enveloped viral entry pathways is shown schematically in Figure 3.28. Good evidence suggests that it passes through an intermediate known as a "hemifusion stalk" (Fig. 3.28, center), in which the two apposed leaflets have fused, but not the distal ones.[219,249,319,356] Hemifused bilayers can then form a "fusion pore" (Fig. 3.28, right) or a structure in which the two distal leaflets create a single bilayer. Bilayers do not fuse spontaneously (eg, concentrated liposomes are quite stable), because the reaction in Figure 3.28 has a high activation barrier, both at the step between the precursor bilayers and the hemifusion stalk and at the step between the hemifusion stalk and the fusion pore. A newly opened pore may revert to a hemifusion structure ("flickering"), and the largest kinetic barrier may be for the step in which the pore dilates rather than reverts.[250,319]

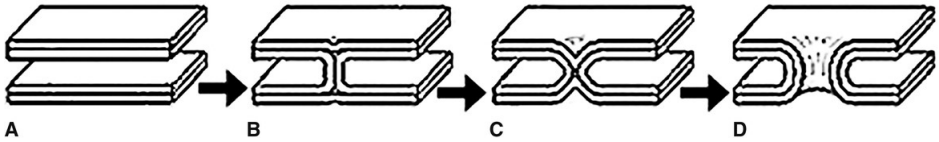

FIGURE 3.28 **Fusion of lipid bilayers**. **A**. Two bilayers. **B**. Hemifusion: only the apposed leaflets have merged, while the distal leaflets on either side remain intact. Formation of a stalk, as shown here, minimizes the area over which the two bilayers must approach each other and hence minimizes the free energy of the transition to the hemifusion intermediate. **C**. Transient opening and closing of a fusion pore. **D**. Opening and widening of the fusion pore.

Viral Fusion Proteins

Viral fusion proteins lower the kinetic barriers to fusion of viral and cellular membranes. They do so by undergoing large-scale conformational rearrangements that lead to tight apposition of the two membranes.[161,162] We can thus distinguish "prefusion" and "postfusion" conformations, as well as potential intermediates. The viral fusion proteins studied at the time this chapter was written fall into three structural classes, usually designated I, II, and III, although the fusion machinery of the otherwise well-studied hepaciviruses appears to be an outlier.[124,216] Common characteristics of fusion by all three classes are insertion of a segment of the fusion protein into the target membrane and refolding of the protein so that this inserted segment and the transmembrane anchor of the protein are adjacent, thereby bringing together cell and viral membranes. The three structural classes probably represent meaningful evolutionary categories, but the phylogeny of the fusion protein is often distinct from that represented by standard viral classification schemes based on replication strategies and polymerase phylogenies, evidently because of extensive genetic exchange. For example, members of the thogotovirus genus of the negative-strand RNA orthomxoviruses have class III fusion proteins, related most closely to gp64 of the dsDNA baculoviruses, while other orthomyxoviruses have class I fusogens.[302] Moreover, eukaryotic cellular representatives of at least two of the classes closely resemble their viral orthologs.[169,257,303]

Class I Fusion Proteins

Unlike members of the two other classes, the class I fusion proteins do not have a consistent overall folded structure. Instead, their defining characteristics are (1) synthesis as a precursor that requires a proteolytic cleavage for activation, either during passage through the trans-Golgi network in the producing cell, while exposed to extracellular proteases during transfer from one cell (or host) to another, while attached at the surface of the target cell, or while engulfed in endosomes; (2) trimeric oligomerization in both pre- and postfusion conformations, based on a central, three-chain, α-helical coiled-coil in the postfusion conformation; and (3) presence of a hydrophobic "fusion peptide" at or near the N-terminus created by the activating cleavage. The fragment C-terminal to the cleavage, with the viral transmembrane segment, is the fusogen; the fragment N-terminal to the cleavage is often a receptor-binding structure, which generally dissociates when suitably triggered, releasing its grip on the C-terminal fragment, liberating the latter to undergo its fusogenic conformational change. The final, postfusion structure is a "trimer of hairpins," as described for three examples below. The N-terminal fragment can have a wide variety of folded structures, even among viruses of a single family.

Influenza A Virus HA

Influenza A virus HA (Fig. 3.29) is the best studied class I fusion protein and one of the simplest.[412] Its properties illustrate most of the features of class I fusion-protein structures and many of the essential features of fusion proteins in general. HA_0, the uncleaved precursor, and HA_1/HA_2, the cleavage product, are almost identical in structure, except for a local shift that tucks the fusion peptide (the N-terminus of HA_2) between the splayed helices of the central coiled-coil (Fig. 3.29B). This local transition makes the cleaved form substantially more stable than the uncleaved precursor.[361] Unless induced to refold by lowered pH or by heat, HA_1/HA_2 is stable. Mutations that shift the midpoint pH of the conformational transition are at widely distributed positions,[94] suggesting that overall charge distribution determines the transition point, rather than a defined trigger (eg, titration of one or more specific residues). The HA_1 "head" is a globular domain at the end of the molecule that faces away from the virus particle. It has a binding pocket (Fig. 3.29B and C) for the receptor, sialic acid.[402]

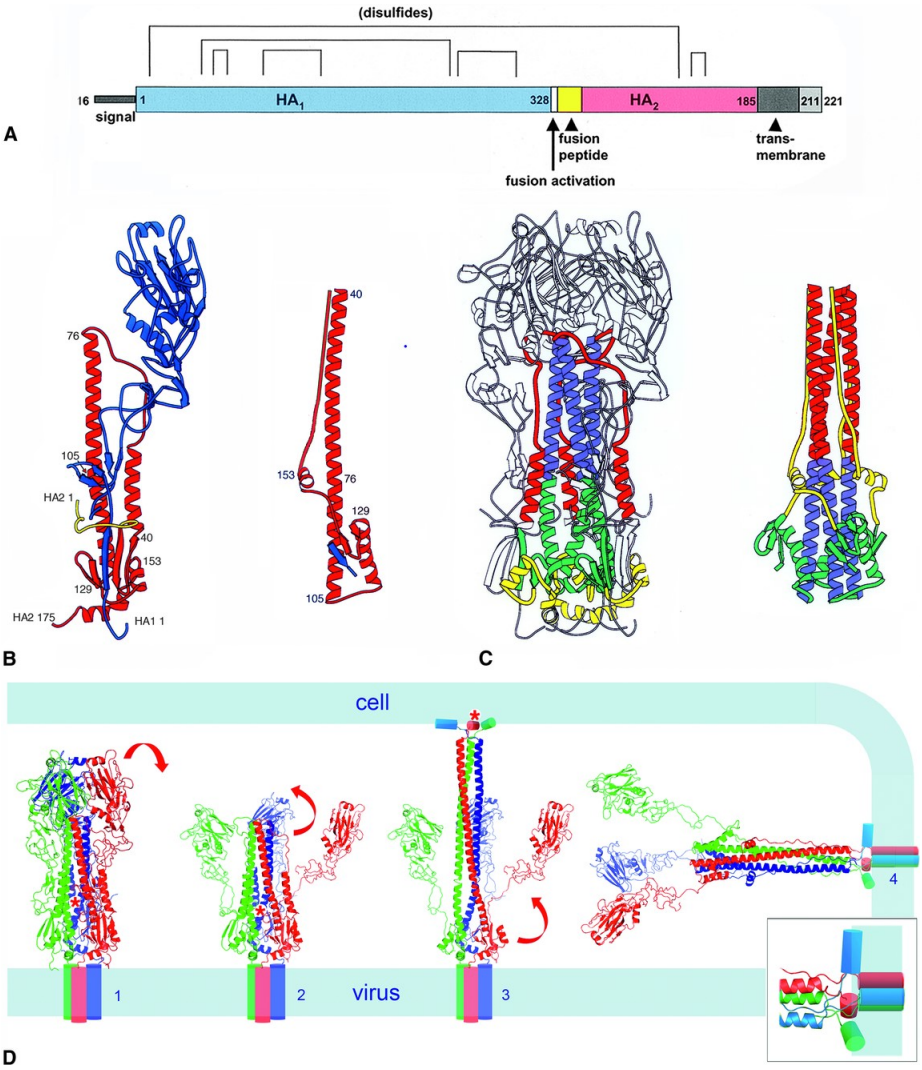

FIGURE 3.29 **Influenza virus hemagglutinin (HA): structure and fusion-promoting conformational changes**. **A**. The HA polypeptide chain. HA_1 in *blue*; HA_2 in *yellow* (fusion peptide), *red* (remainder of ectodomain), and *gray* (transmembrane and internal segments). The position of the fusion activation cleavage between HA_1 and HA_2 is a narrow white stripe, indicating excision of a single residue in many cases. The location of disulfide bonds is shown schematically above the bar. Residue numbers correspond to positions in the HA of strain X-31. **B**. The HA ectodomain monomer, in prefusion and postfusion (the latter, HA_2 only) conformations to the *left and right*, respectively. HA_1 in *blue*; HA_2 in *red*. The postfusion HA_2 structure illustrated here lacks the fusion peptide as well as additional residues at both ends of the chain. **C**. The HA ectodomain trimer, in prefusion and postfusion (the latter, HA_2 only) conformations, to the left and right, respectively. HA_1 in *black and white*; HA_2 in colors showing various segments of the ectodomain, so that their reconfiguration during the transition from the pre- to the postfusion structure is evident by comparison of the pre- and postfusion structures. Note the loop to helix transition in the C-terminal portion of the red segment (for review, see Ref.[362]). **D**. Scheme for the coupling of the fusion-promoting conformational change in HA to the fusing membranes. Stage 1: prefusion conformation. *Red asterisk* shows position of fusion peptide at N-terminus of HA_2. Engagement with a sialic-acid receptor in the target membrane is not shown. Stages 2 and 3: transition to an extended intermediate, in which the three fusion peptides of the trimer associate tightly with the target-cell membrane. The fusion peptide is shown schematically as an amphipathic helix in the membrane surface—the actual structure is probably an amphipathic helical hairpin.[242,243] Proton binding at low pH dissociates HA_1 from HA_2, although the two fragments remain tethered by a disulfide bond. Stage 4: during the transition of HA_2 from intermediate to postfusion conformation, the fusion peptide and transmembrane segment come together, thereby bringing the two membranes close enough to fuse. The final, postfusion conformation of HA_2 is locked in place by the "cap," shown in the *inset*, in which residues near the C-terminus of the HA_2 ectodomain interact with residues between the fusion peptide and the long, central α-helix.[68]

The connection between the HA ectodomain and the helical transmembrane segment has a hinge point, and the ectodomain can "lean" in various directions with respect to the membrane lipid bilayer.[28] On virions, however, the density of HA trimers studding the surface of the particle ensures an "upright" orientation, with all three receptor-binding pockets facing outward. The hinge is selectively sensitive to protease cleavage: prolonged incubation of virions with bromelain releases stable, intact, in general trimeric ectodomain, the form designated BHA (bromelain-cleaved HA) and first crystallized.[410]

Rearrangement of HA induced by proton binding (Fig. 3.29D) has three essential features.[44] One is separation of the HA_1 heads to allow a central coiled-coil to form.[27] A disulfide between a cysteine near the N-terminus of HA_1 and a cysteine near the C-terminus of the HA_2 ectodomain prevents complete dissociation and probably allows the heads to separate transiently, even at neutral pH. A second key feature of

the transition is ejection of the protected hydrophobic "fusion peptide" (Fig. 3.29D, transition from stage 2 to stage 3) from its pocket near the threefold axis of the trimer. The three fusion peptides contact each other, and the ejection is probably cooperative. Augmentation of the central coiled-coil by residues from the loop between the shorter "helix A" of HA_2 and the long central "helix B" may help pull the fusion peptide away from its extensive contacts in the cleaved, prefusion structure. A third key feature is folding back of the fusion protein (HA_2) so that the N-terminus (the fusion peptide) and the C-terminus (the viral membrane anchor) come together (Fig. 3.29D, transition from stage 3 to stage 4).[44,68,362] A likely intermediate state, for which there is good experimental evidence for fusion proteins of all classes,[49,100,129,164] is an extended structure with the fusion peptide buried in one membrane and the anchor in the other (Fig. 3.29D, stage 3). Zipping up of the C-terminal part of the HA_2 ectodomain along the core of this "prefusion intermediate" will cause the transmembrane anchor and the fusion peptide to approach each other. Formation of an intricate "cap" on the three-helix core of HA_2 probably snaps the refolded structure in place (Fig. 3.29D, inset).[68] Note that the zipping up process cannot be symmetrical, because the trimer would otherwise encase itself in lipid. The C-terminal outer-layer segments are long enough, in their unfolded state, to reach around the core of the refolding trimer. Contacts between the three outer-layer chains in the refolded structure are minimal, so that the three can zip up independently and at different rates.

The postfusion conformation of HA is a "trimer of hairpins." The N- and C-termini of each subunit lie at the same end of the elongated protein, and the polypeptide chain traverses the length of the molecule just twice—once from the N-terminus to the distal end, and once back to the C-terminus—with some modest complexity in the distal loop. The "inner core" is a trimeric coiled-coil; the "outer layer" of each subunit is largely extended chain, with a short helical segment. Other class I fusion proteins have the same postfusion characteristics (Fig. 3.30), the structure in the case of HIV-1 being particularly simple, as both inner core and outer layer are helical[29,61,246,404] (Fig. 3.30A). The two helices are sometimes designated HR1 or HRA and HR2 or HRB ("helical region 1" or "A" and "helical region 2" or "B," respectively), but the postfusion HA structure illustrates that identification of two helical regions may not always be informative. Moreover, a major part of the central coiled-coil in postfusion HA_2 is not helical in the prefusion trimer—another reason why "HR1" and "HR2" can be misleading designations. That is, no inferences can be drawn about the prefusion structures of these proteins from their trimer of hairpins postfusion conformers. Note further that in influenza HA, the N-terminal parts of HA_2 are on the outside of the spike in the prefusion conformation and on the inside in the postfusion conformation (Fig. 3.29). The protein turns itself inside out during the refolding.

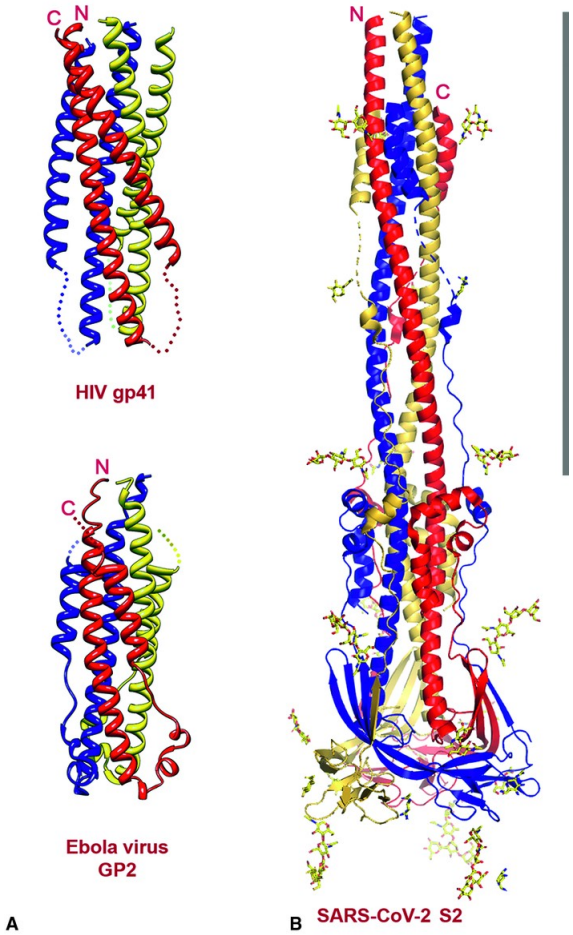

FIGURE 3.30 **Postfusion conformations of class I fusion-protein ectodomains**. **A**. Comparison of HIV-1 gp41 and Ebola virus GP2.[61,403,404,430] The fusion peptide extends from the N-terminus of the trimeric bundle; the transmembrane segment is at its C-terminus. Compare with the postfusion conformations of influenza virus HA2 in Figure 3.29C and of the paramyxovirus hPIV3 F2 protein in Figure 3.33B. The HIV gp41 structure is particularly simple: a six-helix bundle with a relatively short loop (*dotted lines*) between the inner (N-terminal) and outer (C-terminal) helices (HR1 or HRA and HR2 or HRB, respectively). **B**. Full postfusion structure of S2 from SARS-CoV-2, including the membrane-inserted fusion peptides (fp: a helical hairpin), the transmembrane anchors (tm), and a short helical segment of the cytoplasmic tail (ct), labeled for the red subunit. The N-terminus of the red subunit is indicated (the S2' site). Transparent blue region represents approximate extent of the membrane bilayer after fusion. *Gray bar* indicates the region ordinarily designated as HR1 and HR2 (see Refs.[376,441]). About 200 residues of S2 intervene between the HR1 and HR2 segments, most of them folded essentially as in the prefusion conformation. From Shi W, Cai Y, Zhu H, et al. Cryo-EM structure of SARS-CoV-2 postfusion spike in

membrane. bioRxiv/10.1101/2022.12.05.519151v1. Ref.[354a].

The influenza B virus HA is very similar in structure and properties to that of influenza A.[108] The influenza C and D orthologs, known as HEF (hemagglutinin-esterase-fusion) proteins, include an esterase activity that hydrolyses their 9-O-acetylated sialic acid receptors (Fig. 3.31A). Comparison of the influenza C HA with the influenza A and B HA structures shows that the latter have the N-terminal and C-terminal parts of the esterase domain ("vestigial esterase") but lack the central part with catalytic activity.[334]

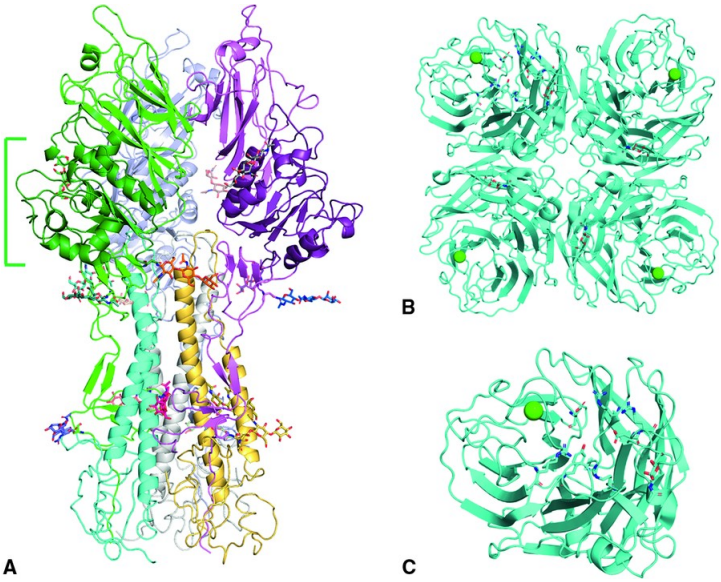

FIGURE 3.31 **HEF and NA**. **A**. Influenza C hemagglutinin-esterase. The esterase domains of the green and purple HA1 fragments are in darker shades of the same color, and the *green bracket* covers the extent of the former. The esterase, a serine hydrolase, cleaves the acetyl group from the C9 position of terminal 9-O-Ac-Neu5Ac residues, removing the viral attachment site.[170] Compare the structure with influenza A HA in Figure 3.29. From PDB 1FLC (Rosenthal PB, Zhang X, Formanowski F, et al. Structure of the haemaggluntinin-esterase-fusion glycoprotein of influenza C virus. *Nature*. 1998;396:92-96. Ref.[334]). **B**. Influenza A neuraminidase tetramer. The *green spheres* are structural Ca^{2+} ions. Some structures also show a Ca^{2+} on the fourfold axis. From PDB 2HTY. **C**. Magnification of one subunit, showing residues in the active site as sticks. (Russell RJ, Haire LF, Stevens DJ, et al. The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. *Nature*. 2006;443:45-49. Ref.[341]).

HIV Env

HIV-1 Env, synthesized as a heavily glycosylated, trimeric, gp160 precursor and cleaved to gp120 and gp41, is the sole HIV-1 surface protein (reviewed in Ref.[65]). The structure of gp120/gp41 on the virion surface, determined at low resolution by cryo-ET,[237] has allowed integration of various substructures, determined at much higher resolution by x-ray crystallography,[200,220,296] cryo-EM,[224,248] and NMR[103,127,308,309,417] (Fig. 3.32). The general organization resembles that of influenza virus HA, but with considerably more elaborate details. The processing cleavage, like the cleavage of HA, does not by itself lead to any large-scale reorganization, but it releases a constraint on conformational dynamics and enables gp41 to refold when sequential binding of receptor (CD4) and coreceptor (either CCR5 or CXCR4, depending on isolate) leads to gp120 dissociation.[65,267] Five variable loops (designated V1 through V5) decorate the gp120 surface,[220] enabling rapid antigenic variation and continuous escape from the on-going B-cell response in an infected host. The bipartite domain structure of gp120 allows CD4 binding to stabilize an initial transition to a more open structure, in which the three clustered V1-V2 loops at the apex of the trimer move apart, exposing the V3 loops to make important contacts with the coreceptor[238,289,295,352,400,418] (Fig. 3.27).

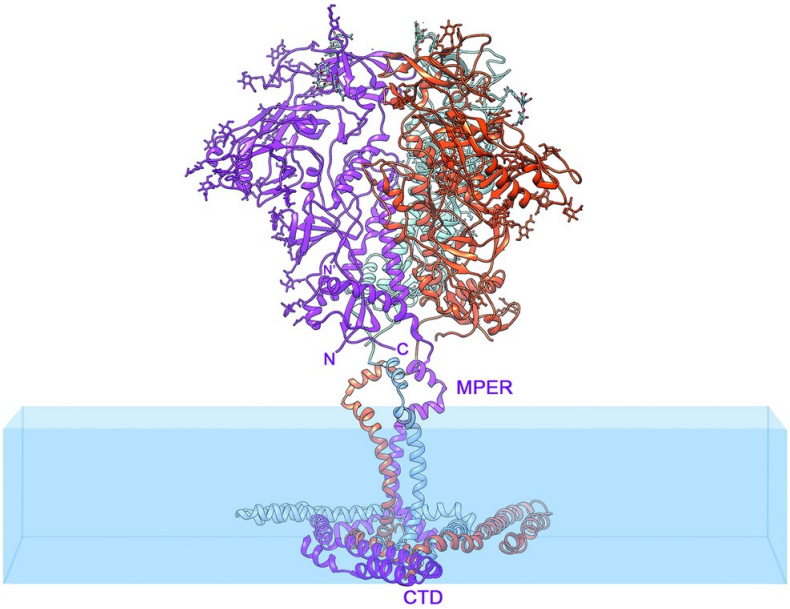

FIGURE 3.32 **HIV Env**. Composite structure of HIV, based on the structure of an unmutated, fully glycosylated, full-length Env extracted directly (in nonionic detergent) from membranes by association with the Fab of human monoclonal PG16 (Fab not shown) (PDB ID: 6ULC)[291] and NMR studies of constructs comprising the membrane-proximal external region (MPER), transmembrane helix (TM), and cytoplasmic domain (CTD) (PDB: 7LOI).[103,127,309] The approximate extent of lipid bilayer is shown (semitransparent *blue* block). The N and C termini of gp120 are marked on one subunit (*magenta*). The first ordered residue at the N terminus of gp41 of the same subunit is labeled N′; the fusion peptide immediately N-terminal to the N′ position is not ordered in the cryo-EM structure.

As in HA, a relatively flexible connection between the HIV-1 Env ectodomain and the membrane-associated components allows the trimeric "spike" to adopt a range of orientations with respect to the membrane bilayer.[291] The membrane-associated structures of gp41 are substantially more extensive than those of HA_2: they include a trimeric "membrane proximal external region" (MPER)[127]; the transmembrane helices, which are also trimer clustered[103]; and a long, elaborately folded C-terminal segment[308,309] (Fig. 3.32). Truncation of the C-terminal segment alters some aspects of the antigenicity of the ectodomain, indicating a coupling between the two, probably through constraints on conformational dynamics.[67] Dissociation of gp120 allows gp41 to refold, ultimately to a rather simple, six-helix bundle (a trimer of helical hairpins: Fig. 3.30A) from its relatively more complex set of shorter helices in the prefusion conformation.[29,61,404]

Paramyxovirus and Pneumovirus F

The fusion (F) proteins of paramyxoviruses and pneumoviruses[69,81,221,420,421] (Fig. 3.33) have a cleavage site just N-terminal to a fusion peptide, which resembles (in being hydrophobic and relatively glycine-rich) the fusion peptides in gp160 and HA_0. Cleavage is essential for fusion activity, but not for the fusion-promoting conformational change, and cleavage does not lead to dissociation of any part of the protein, as it does for HIV-1 Env. In the mushroomlike, prefusion conformation of parainfluenza virus 5, a three-strand α-helical coiled-coil, the stem of the mushroom, is the C-terminal part of the ectodomain. It connects directly (in the intact protein) to the transmembrane segment. The connection between the coiled-coil stem and the head of the mushroom is an ordered, but very extended, stretch of polypeptide chain, which wraps around the outside of the globular cap. The cap also presents a groove to accommodate the fusion peptide. A separate protein (designated HN, H, or G in various paramyxoviruses) binds receptor and triggers the conformational rearrangement of F (reviewed in Ref.[33]; see Chapter 12, volume 1). Its absence may account for the requirement of an ectopic restraint to keep even the uncleaved protein from refolding to a postfusion-like conformation.[421] In the refolded state, a long, three-strand coiled-coil forms from segments (all C-terminal to the cleavage site) that are part of the globular "cap" in the prefusion structure.[420] The C-terminal coiled-coil comes apart so that the C-terminal helices can fold back up along the outside of the newly formed coiled-coil. The length of the connection between the globular domains and these helices, which probably dissociate, unfold, translocate, and refold as they zip along the coiled-coil core, allows sufficient flexibility for this transition. None of the trimer contacts in the prefusion state are fully conserved in the postfusion structure, raising the possibility of a monomeric intermediate, but the overall geometry does permit the assembly to refold as a trimer without such dissociation. The structure of the F protein of respiratory syncytial virus (RSV), a pneumovirus, closely resembles that of PIV5, with a different organization of the C-terminal helices and a more deeply buried fusion peptide in the cleaved form.[256]

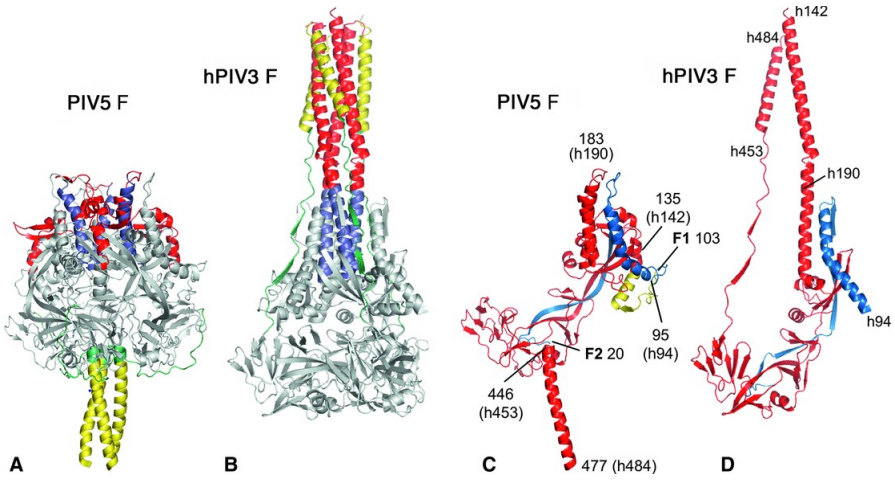

FIGURE 3.33 **The paramyxovirus fusion protein (F)**. **A**. The ectodomain trimer of parainfluenza virus 5 (PIV5 also known as SV5) F in its prefusion conformation. F1 is in black and white; F2, in color. The order of colors corresponds to the order of colors in influenza HA_2 in Figure 3.26C. The viral membrane would be at the bottom of the figure in panel **(A)**: the polypeptide chain of F2 enters the membrane immediately following the *yellow* segment, which forms a three-chain coiled-coil in the prefusion conformation. **B**. The postfusion conformation of F from another paramyxovirus, human parainfluenzavirus 3 (hPIV3). Color scheme as in **(A)**. The fused membranes would be at the top of the figure in panel **(B)**. Note that the red and blue segments toward the N-terminus of F2 have refolded into a three-chain coiled-coil, projecting the fusion peptide (not shown) toward the "top" of the trimer. Compare these segments with those of corresponding color for influenza virus HA2 in Figure 3.26C. Also note that the *yellow* segments at the C-terminus of the F2 ectodomain no longer form a coiled-coil, but rather align along the outside of the coiled-coil generated by the (*red* and *blue*) N-terminal region. **C, D**. Pre- and postfusion conformations of monomers, with F1 in *blue*, F2 in *red*, and fusion peptide (N-terminus of F2) in *yellow*. Numbers in **(C)** correspond to PIV5 F; numbers with "h" in **(C)** and **(D)**, to hPIV3 F. (Figure courtesy Ted Jardetzky, Stanford University.)

Class II Fusion Proteins

The defining characteristics of fusion proteins in this class are a three-domain subunit (domains I, II and III or DI, etc.), with an internal, hydrophobic "fusion loop" at the tip of the elongated DII; association with a second, "chaperone" protein that protects the fusion loop; and formation of a stable trimer in the postfusion state, with the three fusion loops and the three C-terminal, transmembrane anchors clustered at one end (Fig. 3.12). Found initially on flaviviruses and alphaviruses, both of which are compact, icosahedrally symmetric particles, class II proteins are also the fusion proteins of bunyaviruses and hence very widespread. They are known as E1 and E in alphaviruses and flaviviruses, respectively, and as Gc in bunyaviruses; the chaperones, as p62, prM, and Gn. Cleavage of p62 or prM generates E3 + E2 or pr + M, with release of E3 or the pr fragment. Gn does not require cleavage to allow Gc to mediate low-pH triggered fusion and viral entry. Although roughly similar in their overall, multidomain organization, in a common β-ribbon core, and in their association with the fusion protein, the chaperones of different virus families have otherwise divergent structures.[151] (An exception to this statement is the polypeptide-chain fold of alphavirus E2 and hantavirus Gn.[150])

The flavivirus E protein tiles the surface of the mature virion as a tightly associated dimer, in the herringbonelike pattern described above[215] (Fig. 3.12). On an immature particle, before cleavage of prM (the chaperone), E forms heterodimers with prM rather than homodimers with itself.[215] The prM-E heterodimers cluster as asymmetric trimers at neutral pH (eg, after budding into the ER) but rearrange into dimers at the somewhat lower pH of the trans-Golgi network, where furin cleaves prM. The "pr" domain, which covers the fusion loop, dissociates only when the particle again reaches neutral pH as it emerges from the cell[227,423,424]; the fusion loop at the tip of domain II then packs against domain III of the dimer partner. The short ectodomain of the residual M protein that remains lies between E1 and the membrane.

Alphaviruses have a T = 4 icosahedral arrangement of E1-E2 heterodimers, clustered into threefold protrusions by E2 contacts (Fig. 3.23B). E2 has three, Ig-like domains; the second of these covers the fusion loop of E1. The C-terminal tip of the E1 transmembrane helix contacts a core-protein subunit. The MXRA8 adhesion molecule, the receptor for most arthritogenic alphaviruses, binds at the interface between E1 and E2,[21] and hence both proteins in their native heterodimeric pairing participate in receptor recognition. The LDL-related LDLRAD3 receptor of VEEV inserts into a cleft between threefold-related E1-E2 heterodimers, also contacting both proteins.[22]

The organization of bunyavirus Gn-Gc on the surface of virions varies from one group of this large family to another.[151,183] Crystal structures of Gc from Rift Valley Fever virus (RVFV),[102] severe fever with thrombocytopenia syndrome virus (SFTSV),[156] Puumala virus,[411] Hantaan virus,[149] and a number of others, in either pre- or postfusion conformations, show the familiar class II structure. Partial Gn crystal structures and predicted folds are more variable, but in general, there are three domains, organized by a structurally conserved β-ribbon.[151] Despite the T = 12 arrangement on phenuivirus particles (eg, RVFV), the particles deform readily, limiting the resolution of cryo-EM structures.[157,182] Fitting crystal structures into a 13-Å resolution reconstruction of the virion indicates that the first two domains of Gn occupy the prominent rings of five- and sixfold density seen in the reconstruction and that the second domain shields the fusion loop of Gc.[157] The heterodimer interaction is reminscent of the alphavirus arrangement, even though the domains have somewhat different folded structures, but the surface clustering of the heterodimers is quite different. The hantaviruses are pleomorphic: four Gn-Gc heterodimers cluster into a fourfold complex that packs tightly on the surface of the particle but not in a locally well-ordered array.[183,228]

The postfusion trimers that form at acidic pH have very similar conformations for all class II fusion proteins (Figs. 3.12 and 3.34). The conformation of the fusion loop is very similar across essentially all group II fusion proteins, including a disulfide that locks it in a relatively unusual configuration and a projecting tryptophan side chain. A pocket adjacent to the fusion loop, conserved in most group II fusion proteins,

accommodates the headgroup of a glycerophospholipid in the postfusion trimer of RVFV Gc.[148] It probably confers some lipid headgroup affinity in other cases as well. Electron microscopy of postfusion trimers bound to liposomes show that the fusion loops insert part way into the outer leaflet of the target membrane, consistent with the position of the headgroup binding site. Figure 3.34 illustrates likely steps in the flavivirus fusion process, deduced from comparison of the pre- and postfusion conformations of dengue- and TBE-virus fusion proteins.[163,281,386,416]

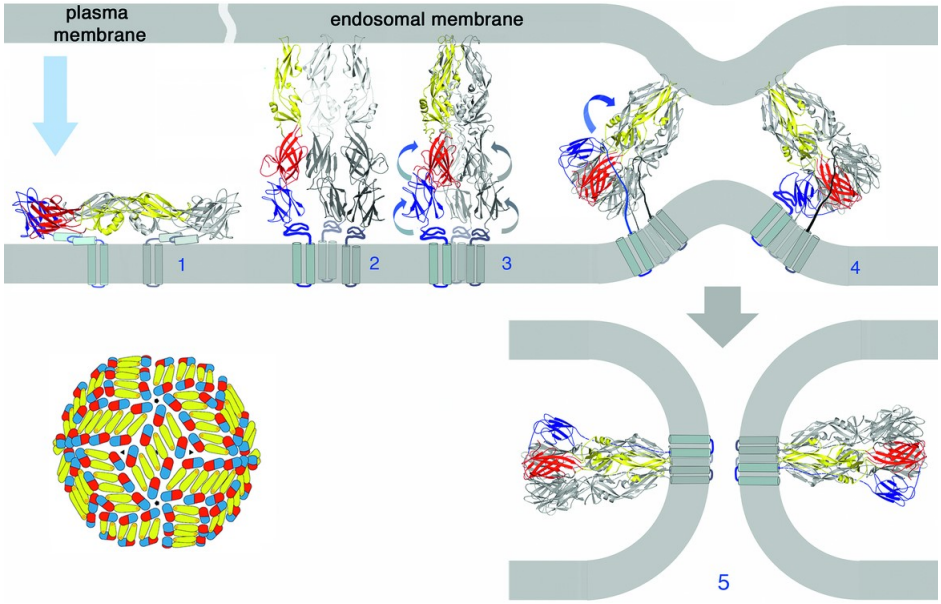

FIGURE 3.34 **Flavivirus envelope protein, E, and fusion-promoting conformational changes**. Diagram *lower left*: dimer-clustered packing of E on the virion surface. The three domains of each protein ectodomain are in *red*, *yellow*, and *blue*. Numbered sequence, starting on top row: likely series of conformational states of E and their links to viral and cellular membranes. Structures for states (1) and (5) are known (Fig. 3.12C); those for states (2)-(4), shown as monomers only (because maintenance of trimer state during the fusion-promoting transition is uncertain), are inferred from indirect data.[64,262] One subunit in each dimer or trimer is colored as in the schematic; its partner(s) are in *gray*. (1) E dimer on the virion surface. The ectodomain terminates in a helical hairpin called the "stem" (*light blue*) on the surface of the viral membrane (*lower gray bar*) and connects with a transmembrane helical hairpin. *Blue arrow* from above symbolizes a receptor interaction with domain III (*blue*). (2) Exposure to low pH (following endocytosis and acidification of endosome) dissociates the E dimer, allowing the subunits to project outward, so that the fusion loops (tip of the *yellow* domain II) encounter the endosomal membrane (*upper gray bar*). (3) Initial trimer association, requiring rearrangement across the surface of the virion. *Arrows* show presumed pattern of folding back; *upper arrows*: domain III (*blue*) flips over against domain I (*red*); lower arrows: the stem refolds to "zip up" alongside the trimer clustered domain II (*yellow*). (4) Stem continues to reorganize (asymmetrically), pulling the two membranes together. The fusion loops must be firmly anchored in the target membrane. (5) Formation of a fusion pore allows the refolding to finish, so that all three stems and all three fusion loops cluster together, restoring full threefold symmetry.

Class III Fusion Proteins

Class III fusion proteins mediate penetration of viruses as distinct as rhabdoviruses,[18,19,25,330,332] herpesviruses,[12,165,240,394] and thogotoviruses,[302] as well as insect baculoviruses.[11,202] There appears to be no proteolytic cleavage required for priming, and the fusion-inducing conformational change is in at least some cases reversible[331]: virions inactivated by prolonged incubation at pH < 6 can be reactivated by returning the pH to neutral or above, and both conformations of the multidomain, trimeric protein can be obtained from the same protein preparation.

VSV-G, the only protein on the surface of the virion, has two hydrophobic loops that can interact with membrane lipids[113] (Fig. 3.35). The connectivity of the strands joined by these fusion loops is different from the connectivity in domain II of the class II proteins (ie, the domains themselves have different folds), but the general picture is quite similar: hydrophobic residues (including at least one tryptophan) are displayed on tightly structured loops at the tip of an elongated domain. In the prefusion conformation of VSV-G, these domains face the viral membrane around the periphery of the trimer.[332] In the postfusion conformation, they cluster around the threefold axis[330](Fig. 3.35).

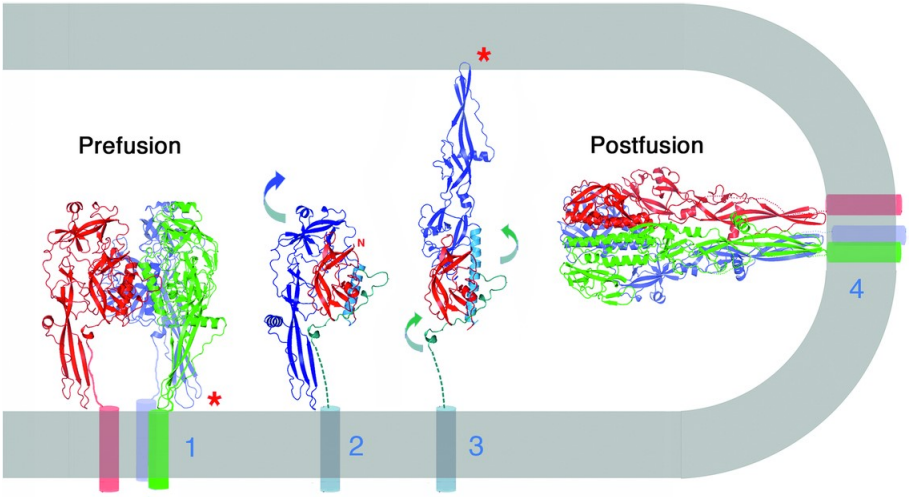

FIGURE 3.35 **VSV glycoprotein, G: structure and fusion-promoting conformational changes**. Sequence of conformational events as in Figures 3.26 and 3.31.[207] (1) Prefusion trimer of VSV G on the virion surface.[332] Subunits colored in *red*, *blue*, and *green*. *Red asterisk*: fusion loops of the green and blue subunits. (2) Conformation of one subunit of the trimer in (1), now colored to show a central, red, domain and a set of peripheral, blue domains. An axial helix (*lighter blue*) joins part of the central domain to the set of peripheral domains. In the low-pH triggered rearrangement, the blue regions reorient with respect to the red domain (*arrow*). (3) Presumed initial rearrangement, in which the domain bearing the fusion loops projects toward the cellular (endosomal) membrane, into which the fusion loops insert. The axial helix remains, augmented by a segment derived from its connection to the peripheral domains. *Arrows* indicate likely reorganization that follows: the central domain flips over and the "stem" at the C-terminus of the ectodomain zips up, bringing together the two membranes. (4) Formation of a fusion pore (not shown explicitly—compare the last two stages in Fig. 3.29) allows the three sets of fusion loops and the membrane-proximal segments of the ectodomain to cluster. Part of the stem rearrangement includes formation of helical segment, which forms a six-helix bundle with the central-region helix. The conformational details thus have features of both "class I" fusion proteins (formation of a six-helix bundle) and of "class II" fusion proteins (preconfigured, internal fusion loops that insert into the target membrane).[330]

In the rhabdovirus G protein, a core domain contains residues from the N-terminal segment of the polypeptide chain and residues from near the C-terminal part of the chain: it is a framework around which the rest of the molecule reorients (Fig. 3.35). Two other domains form a jointed, two-part fusion machinery. The result of their rotations relative to the core domain (and to each other) is to move the fusion loops away from the viral membrane and toward the target membrane. In a likely extended intermediate conformation, the C-terminal segment still connects toward the virus, while the fusion loops interact with the target. In the fully rearranged, low-pH conformation, the C-terminal segment has zipped up along the fusion domains, much as in the flavivirus fusion transition.

The herpesvirus fusion protein, glycoprotein B (gB), looks like an elongated version of VSV-G.[12,165,240] This unexpected similarity between fusion proteins of a DNA virus and a negative-strand RNA virus has allowed information about one protein (eg, the identification of the rhabdovirus fusion loops) to be carried over to the other.[158] The only high-resolution, prefusion structure of a gB determined at the time of writing is that of human cytomegalovirus.[240] Stabilization of the prefusion conformation required introducing designed mutations—inspired by strategies useful for structural studies of HIV-1 Env,[200,203,248] paramyxovirus F,[178] and coronavirus spike.[177] The gB conformational transition is triggered not by changes in pH but rather by receptor binding to another surface protein, gD.[53,213] A binding-induced conformational change in gD leads to the transition of gB to the postfusion conformation, with participation of yet another protein, the gH/gL heterodimer.[77,252]

Other Viral Glycoproteins

The influenza viruses and paramyxoviruses, both of which bud at the cell surface, require a receptor-destroying enzyme to allow them to escape rebinding to the cell from which they have emerged.[290] The receptor for influenza A and B viruses is sialic acid, and the receptor destroying enzyme is the neuraminidase (NA). The structure of NA (Fig. 3.31B), a six-blade β-propeller, resembles that of catalytic domains of various bacterial sialidases. NA is both the target of widely used influenza inhibitors (oseltamivir and zanamavir),[397] which block spread of the virus at early stages of infection, and, like all viral surface proteins, NA is a viral antigen. The receptor destroying function is a domain of the HEF protein of influenza C and D viruses (Fig. 3.31A) and a domain of the receptor-binding hemagglutin-esterase of paramyxoviruses. Like herpesviruses, paramyxoviruses have a distinct receptor-binding protein, which constrains the fusion protein in its prefusion conformation when the two are present together on the virion surface and releases it to refold when receptor binds.

Membrane Fusion

The molecular architectures of enveloped-virus fusion proteins vary substantially, as Figures 3.29-3.36 illustrate, but the events that lead to formation of a hemifusion stalk and ultimately of a fusion pore appear to be essentially the same. Interaction with one or more ligands initiates a conformation change that results in formation of an extended intermediate bridging between viral and host-cell membranes. To overcome the kinetic barrier to stalk formation, a small number (~2-5, see below) of adjacent, extended intermediates fold back toward the postfusion conformation. The free energy recovered from this favorable transition is enough to pull the membranes together over the small area enclosed by the narrow "palisade" of collapsing intermediates, and the apposed outer leaflets at the tips of the nipplelike bilayer distortions then merge. To reach the final, postfusion conformation, the fusion peptides and transmembrane anchors must come together completely, requiring the internal (cytoplasmic) domains of the fusion proteins to pass into a transiently opened, aqueous pore and to drive further pore opening by preventing reclosure.

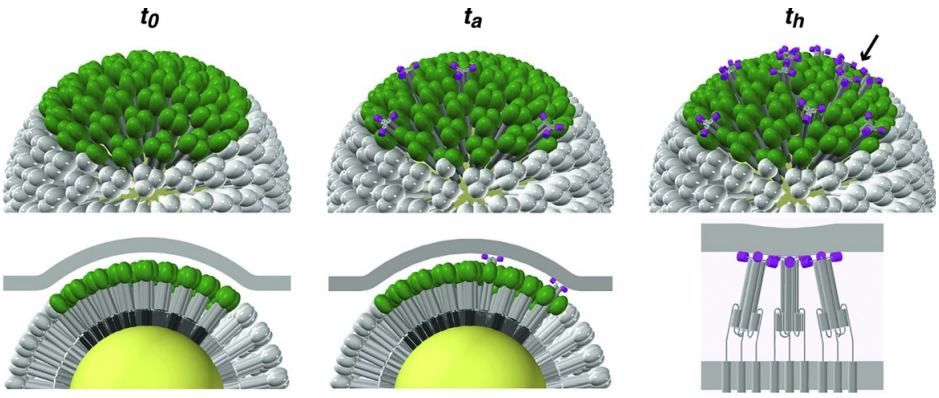

FIGURE 3.36 **Stages of influenza HA–mediated membrane fusion**. Virion dimensions in the diagram are based on a spherical particle, 55 nm in membrane to membrane diameter.[50] There are ~50 HA trimers in the contact area between the virus and the target cell, shown in *green* (HA1) and *gray* (HA2); trimers outside of the contact area are in *white*. t_0: virion engages a target membrane by HA-receptor interactions at neutral pH, but can still move freely relative to the target-membrane area of contact. t_a: as the pH drops after uptake by endocytosis, fusion peptides of HA trimers project outward and engage the target membrane, forming extended intermediates. Isolated extended intermediates do not exert enough force on the membrane to distort it toward a hemifusion stalk. t_h: engagement of fusion peptides from three *neighboring* HA_2 trimers, which can together exert the required distorting force. Rapid folding back ("zippering") of the three HAs leads to hemifusion (see Fig. 3.29D). For t_h, HA1s are left out for clarity from the *bottom panel*, which shows three neighboring trimers about to fold back. (From Ivanovic T, Choi JL, Whelan SP, et al. Influenza-virus membrane fusion by cooperative fold-back of stochastically induced hemagglutinin intermediates. *Elife*. 2013;2:e00333. http://creativecommons.org/licenses/by/3.0/. Ref.[187])

The most detailed picture of these events comes from analysis of *in vitro*, single-virion fusion kinetics of influenza virus[122,187,188] (Fig. 3.36). Initial attachment, through multiple sialic-acid contacts, establishes an extended contact zone between the virus particle and the target membrane. For influenza virus particles, that contact zone includes 50 or more HA trimers, owing to the tight packing of HA on the surface of typical influenza virus particles. When the pH falls below a critical level (about 5.5 for the H3 viruses studied), individual trimers undergo (stochastically) the first steps of the fusogenic conformational change, leading to extended intermediates that span the two membranes, with fusion peptides in the target membrane and TM segments in the viral membrane. To initiate hemifusion stalk formation requires a small number of *immediately adjacent* HA molecules, as nonparticipating HAs between any two refolding ones would interfere with drawing the two membranes close together. The number of required HAs that best fits the observations is about 3,[122,187,188] comparable to estimates from measurements of HA-expressing cells fusing with red blood cells.[93,122] (These numbers are necessarily an average. In an ensemble of fusion events, a few events might occur with just 2 HAs and others with 4, depending on local molecular fluctuations.) Related studies of flavivirus and flavivirus VLP fusion[64] and of VSV fusion[207] have led to similar conclusions, with at least 2 refolding trimers required for flavivirus fusion and between 3 and 5 for VSV.

Penetration by Nonenveloped Viruses

Nonenveloped viruses must breach a membrane to access the cytoplasm or nucleus of a cell, but unlike their enveloped cousins, they cannot do so by membrane fusion. Current experimental observations show three kinds of mechanism by which a nonenveloped particle, bound at the surface of a cell or taken up into an endosome or other internal compartment, translocates itself (or its genome) across the intervening lipid bilayer—ie, "penetrates." The first involves creation of a pore, through which the viral genome is drawn into the cell through some sort of channel. The elaborate injection structures of many bacterial viruses (eg, the T-even bacteriophage) illustrate an extreme version of this class of mechanism, which appears to be the way some single-strand, plus-sense RNA viruses penetrate. The second class, membrane "perforation," requires a more extensive, disruption of a cellular membrane (eg, the membrane of an endocytic vesicle or an endosome), in order to admit the virion, usually in altered form, into the cytosol. The third, found so far only in the parvoviruses, involves exposure of a lipase that chemically disrupts the surrounding membrane. For all three classes, binding of a receptor, coreceptor, or some other ligand induces a conformational change in the virus particle, with consequent exposure of previously buried, hydrophobic, or enzymatically active structures.

Picornavirus particle expand transiently when the bind receptor, releasing not only the N-terminally myristoylated VP4[76] but also exposing a hydrophobic N-terminal segment of VP1.[42,125,137,153,155] Myristoyl groups target proteins to membranes, and it is logical to suppose that exposure of the myristoylated peptide causes it to associate with membranes and ultimately to contribute to penetration. In keeping with this supposition, picornavirus VP4 makes pores in membranes.[95,297] Images and reconstructions from low-resolution cryo-EM and cryo-ET of poliovirus particles bound to membrane-anchored receptors suggest that some combination of the released VP4 and the exposed segment of VP1 form a channel, through which the genomic RNA can pass into such pores.[34,42,43,173,388]

For another class of single-strand, plus-sense RNA viruses, there is high-resolution evidence for the formation of such a tubelike projection. When a feline calicivirus particle decorated with a soluble ectodomain of its receptor, feline junctional adhesion molecule A, is exposed to acidic pH, an internal protein, VP2, present in 12 copies, oligomerizes into a tubelike projection at a single threefold position on the particle surface[80] (Fig. 3.37). The hydrophobic N-terminal tips of VP2, disordered in the absence of a membrane, point away from the virion surface; the logical inference is that when endocytosis of the receptor-bound virion carries it into an endosome, the VP2 tips insert into the endosomal membrane. The calicivirus and picornavirus models both resemble "just on time" assembly of a phage tail–like structure. RNA delivery would then require a mechanism for destabilizing secondary-structural elements in the RNA. One candidate "helicase" would be a ribosome or ribosome-associated factors, by analogy with an *in vitro* uncoating mechanism found for certain positive-strand RNA plant viruses. For those viruses, exposure of the 5′ end of the RNA (eg, through expansion of the virion induced by intracellular ionic conditions) leads to association of ribosomes with the still largely packaged RNA genome; progress of the ribosome along the message-sense genome and sequential formation of a polysome appears to uncoat the RNA cotranslationally.[16,38,354,355,413] A similar "striposome" mechanism could, in principle, draw RNA through a membrane pore

as well as through an opening in the viral shell.

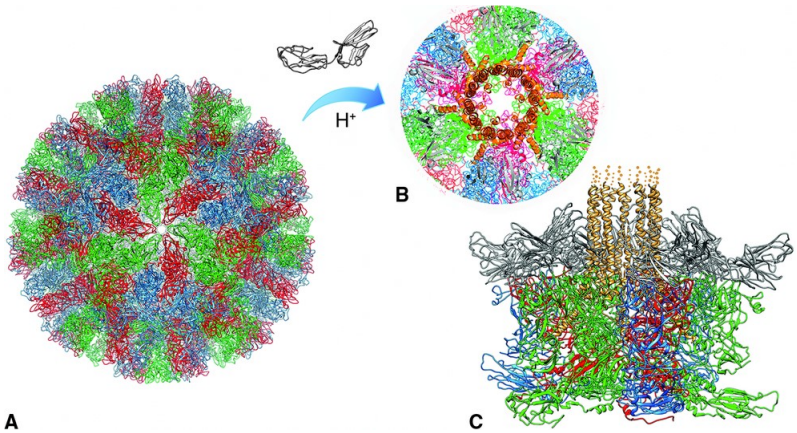

FIGURE 3.37 **Calicivirus-receptor complex at low pH: formation of VP2 portal**. **A**. Feline calicivirus particle (T = 3), viewed along a threefold axis. VP1 subunits in A, B, and C positions colored *blue*, *red*, and *green*, respectively, as in Figures 3.6 and 3.9. Dashed circle shows region of blow-up in B. B, **C**. Binding to receptor (feline junctional adhesion molecule A: fJAM-A) and exposure to pH of endosomes triggers translocation outward of 12 VP2 subunits (*orange*), which form a tubelike structure ("portal"), likely to be a route of RNA exit. An N-terminal region of fJAM-A (two Ig-fold domains, shown in cartoon representation above the *curved arrow*) associates with VP1 dimers, accompanied by local conformational changes that open a channel in the VP1 shell, viewed along the axis of the channel, in **B**, and normal to that axis, in **C**. In the latter view, *dotted lines* show the hydrophobic, N-terminal segments of the twelve VP2 polypeptide chains, likely to insert into a target-cell membrane. From coordinates in PDB files 6GSH and 6GSI. (Conley MJ, McElwee M, Azmi L, et al. Calicivirus VP2 forms a portal-like assembly following receptor engagement. *Nature*. 2019;565:377-381. Ref.[80])

Adenoviruses enter cells by endosomal uptake; penetration proceeds by disruption of the endosome containing the virion.[121,141] The subviral particle admitted to the cytoplasm lacks pentons, as a result of events triggered by receptor and coreceptor binding. Exposure of an internal viral protein, pVI, which depends on the activity of a packaged viral protease, leads to perforation of the endosomal membrane.[357,408] The membrane-disrupting properties of pVI may come from an N-terminal amphipathic α-helix.[269] Following penetration, the partially stripped virion migrates to a nuclear pore, where it disassembles and liberates its DNA for nuclear import.

Like adenoviruses, the dsRNA viruses release into the cytoplasm an intact, roughly 700-Å diameter subviral particle (called the "core" in the case of reoviruses and the "double layered particle" in the case of rotaviruses). This inner capsid particle never uncoats, however, as it contains all the enzymes necessary for mRNA synthesis and modification (see Chapter 11, volume 3). The penetration protein of mammalian orthoreoviruses is the outer-shell trimer, μ1[62,175,281](Fig. 3.27). On the virion, this protein is associated with a "chaperone" subunit, σ3; degradation of σ3 (by proteases in the gut or by cathepsins in endosomes) and autocleavage of μ1 allows μ1 to release a myristoylated, N-terminal peptide (μ1N). The released peptide, up to 600 copies of which could emerge from a single virion, forms membrane pores.[186,431] The autocleavage step is similar to the corresponding step in picornaviruses that separates VP4 from VP2; the correspondence extends not only to pore formation by the released fragment but also to the folded structure of uncleaved μ1, which, like VP1, 2, and 3 of picornaviruses, is a jelly-roll β-barrel subunit (Fig. 3.27). The sequence of events that precedes membrane perforation is quite different for the two types of virus, however.

The attachment/penetration protein of rotaviruses is VP4, which must be cleaved to VP8* + VP5* in order to activate it for entry [84] (see Chapter 12, volume 3). VP4 is not a homolog of μ1, although rotaviruses do have such a homolog, VP6, which appears to have a purely structural role[251] (Figs. 3.12 and 3.38). VP4 is a trimer that projects as an asymmetric, upright "spike,"[350] held in place by the second outer-layer protein, VP7. Only local changes accompany cleavage to VP8* and VP5*, but like class I fusion proteins, the cleaved trimer undergoes a major conformational rearrangement when suitably triggered, inserting a substantial C-terminal segment of the VP5* polypeptide chain into the membrane to which it is attached by the RBD of VP8*[2,106,171,208,422] (Fig. 3.37). A combination of live-cell imaging studies[2,343,344] and high-resolution cryo-EM structures[171,350] has yielded a moderately complete "movie" of the molecular events that accompany rotavirus penetration, but breakage of the vesicular membrane remains (at the time of writing) a molecular blind spot.

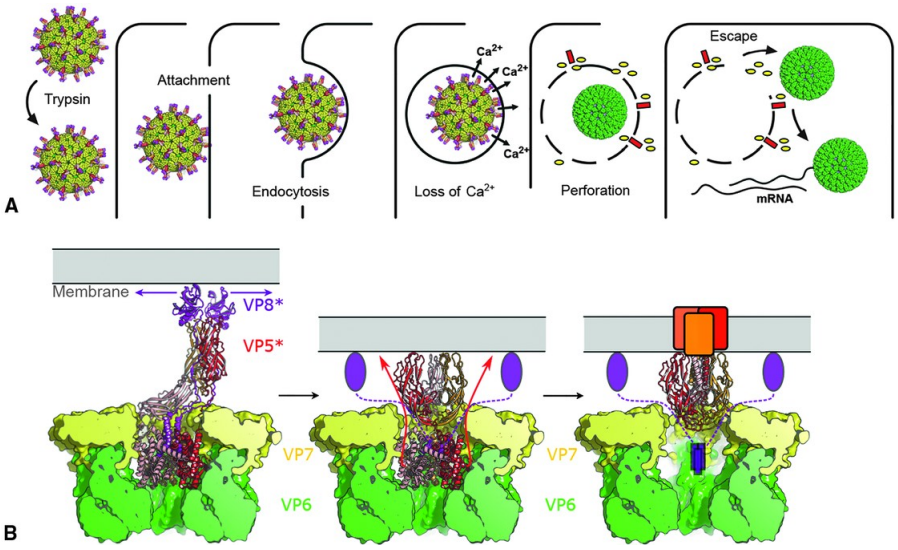

FIGURE 3.38 **Rotavirus entry**. See Figure 3.13 for overview of rotavirus structure. **A**. Scheme for stages of rhesus rotavirus infection of BSC-1 cells.[2,343,344] Tryptic cleavage of VP4 to VP8* (receptor-binding fragment) and VP5* (membrane-interacting fragment) primes the trimeric spike to mediate entry. Receptor binding (the receptor is often part of a glycolipid head group) leads to membrane invagination (clathrin-independent in some cells, but at least sometimes clathrin-mediated in others). Rearrangement of VP5* and its concomitant interaction with the surrounding membrane (details in panel **B**) allow escape of Ca^{2+}; the reduced Ca^{2+} concentration destabilizes VP7, releasing the rearranged VP5* trimer from the particle and allowing (by a mechanism yet to be determined) escape of the DLP into the cytosol. **B**. VP5* rearrangement and membrane insertion. Binding of VP8* lectin domain to glycolipid head group and transient dissociation of lectin domain from projecting part of VP5* (*left panel*) allows capture of hydrophobic loops at the tips of the VP5* domains; membrane insertion prevents reassociation of VP8* lectin domain, which is tethered at its N-terminus into the folded "foot" domain at the C-terminus of VP5* (*center panel*). Unfolding and emergence of foot domains leads to their insertion into the membrane of the cell (*right panel*). The foot-insertion step probably permeabilizes the membrane to Ca^{2+}, leading to loss of Ca^{2+} from the particle following complete engulfment.[343] VP8*, *magenta*; VP5*, shades of *red*; VP7, *yellow*; VP6, *green*. (From Herrmann T, Torres R, Salgado EN, et al. Functional refolding of the penetration protein on a nonenveloped virus. *Nature*. 2021;590:666-670. Ref.[171])

The parvovirus capsid protein has two or three forms, with the jelly-roll structure at their common C-termini and varying N-terminal extensions, determined by alternative splicing and alternative start codons.[260] The two forms in canine parvovirus (CPV), VP1 (~84 kDa) and VP2 (~67 kDa), are present in a ratio of about 5:55.[276] The additional residues in VP1, which extend into the particle interior, include an N-terminal phospholipase 2 (PLP2) domain and a linker segment with a nuclear localization sequence (NLS). From the outside of the particle, there is no apparent difference between VP1 and VP2, and structures known at the time this chapter was written do not show whether the five or six VP1 subunits are clustered or distributed. More copies would leave no room inside the shell for the DNA genome. CPV binds the transferrin receptor and enters the endolysosomal pathway by clathrin-mediated endocytosis.[89,299,300] Structural changes (presumably some expansion) at low pH cause the PLP2 of VP1 to move to the outside of the particle; enzymatic hydrolysis of endosomal lipids allows the particle to escape into the cytosol and move to the nucleus, presumably by importin recognition of the linker-segment NLS. The adeno-associated viruses (AAVs) have different receptors but follow a similar route.

Perspective

The explosion of cryo-EM–derived structures since the last edition in 2014 has illustrated, with many variations, the "principles" previously extrapolated from a handful of model examples. Most critically, it has allowed virologists to apply these principles directly to their virus of interest and to design experiments to relate structure and function in specific detail. In just over 2 years, the structural picture we have of the maturation, antigenicity, immunogenicity, receptor binding, and fusion of SARS-CoV-2 nearly rivals the accumulation of information from over 40 years of work on similar aspects of influenza virus. But it also validates the investment of those 40 years, for without the conceptual foundation established by that work and by studies of other selected viruses, much of the effort on SARS-CoV-2 would have taken far longer. The same is true, with less fanfare, for many other emerging or re-emerging viral pathogens.

What comes next? Many on-going efforts will doubtless accelerate—for example, probing the structural basis of viral antigenicity, in the interest of more rational vaccine immunogen design. Prompt structural attention to newly identified threats to global health will surely facilitate their containment. A more fundamental issue is how to relate the molecular events we can study by direct structural analysis to what happens as a virus encounters a cell. The advances in live-cell imaging and image analysis that combine with cryo-EM and cryo-ET to produce the scheme in Figure 3.38 offer a way to make this connection. Tracking individual virus particles from their initial attachment to a receptor through all the stages of internalization that lead to transcription and replication will help distinguish productive from unproductive entry pathways and resolve ambiguities about events that occur only in intact cells. Direct visualization, with comparable, single-particle resolution, of transfer from producer to recipient cell will be similarly enabling. Future editions will need movies of the structural transformations described here, in the experimentally derived "scenery" of living cells and tissues.

REFERENCES

1. Aathavan K, Politzer AT, Kaplan A, et al. Substrate interactions and promiscuity in a viral DNA packaging motor. Nature. 2009;461:669-673.

2. Abdelhakim AH, Salgado EN, Fu X, et al. Structural correlates of rotavirus cell entry. PLoS Pathog. 2014;10:e1004355.

3. Abrescia NG, Cockburn JJ, Grimes JM, et al. Insights into assembly from structural analysis of bacteriophage PRD1. Nature. 2004;432:68-74.

4. Adrian M, Dubochet J, Lepault J, McDowall AW. Cryo-electron microscopy of viruses. Nature. 1984;308:32-36.

5. Ahi YS, Mittal SK. Components of adenovirus genome packaging. Front Microbiol. 2016;7:1503.

6. Albertini AA, Wernimont AK, Muziol T, et al. Crystal structure of the rabies virus nucleoprotein-RNA complex. Science. 2006;313:360-363.

7. Allison SL, Tao YJ, O'Riordain G, Mandl CW, Harrison SC, Heinz FX. Two distinct size classes of immature and mature subviral particles from tick-borne encephalitis virus. J Virol. 2003;77:11357-11366.

8. Arisaka F, Yap ML, Kanamaru S, Rossmann MG. Molecular assembly and structure of the bacteriophage T4 tail. Biophys Rev 2016;8:385-396.

9. Arranz R, Coloma R, Chichon FJ, et al. The structure of native influenza virion ribonucleoproteins. Science. 2012;338:1634-1637.

10. Atanasova NS, Sencilo A, Pietila MK, Roine E, Oksanen HM, Bamford DH. Comparison of lipid-containing bacterial and archaeal viruses. Adv Virus Res. 2015;92:1-61.

11. Backovic M, Jardetzky TS. Class III viral membrane fusion proteins. Curr Opin Struct Biol. 2009;19:189-196.

12. Backovic M, Longnecker R, Jardetzky TS. Structure of a trimeric variant of the Epstein-Barr virus glycoprotein B. Proc Natl Acad Sci U S A. 2009;106:2880-2885.

13. Badger J, Minor I, Kremer MJ, et al. Structural analysis of a series of antiviral agents complexed with human rhinovirus 14. Proc Natl Acad Sci U S A. 1988;85:3304-3308.

14. Baker ML, Jiang W, Rixon FJ, Chiu W. Common ancestry of herpesviruses and tailed DNA bacteriophages. J Virol. 2005;79:14967-14970.

15. Baker TS, Olson NH, Fuller SD. Adding the third dimension to virus life cycles: three-dimensional reconstruction of icosahedral viruses. Microbiol Mol Biol Rev. 1999;63:862-922.

16. Bakker SE, Ford RJ, Barker AM, et al. Isolation of an asymmetric RNA uncoating intermediate for a single-stranded RNA plant virus. J Mol Biol. 2012;417:65-78.

17. Bamford DH, Caldentey J, Bamford JK. Bacteriophage PRD1: a broad host range DSDNA tectivirus with an internal membrane. Adv Virus Res. 1995;45:281-319.

18. Baquero E, Albertini AA, Gaudin Y. Recent mechanistic and structural insights on class III viral fusion glycoproteins. Curr Opin Struct Biol. 2015;33:52-60.

19. Baquero E, Albertini AA, Raux H, et al. Structure of the low pH conformation of Chandipura virus G reveals important features in the evolution of the vesiculovirus glycoprotein. PLoS Pathog. 2015;11:e1004756.

20. Basnak G, Morton VL, Rolfsson O, Stonehouse NJ, Ashcroft AE, Stockley PG. Viral genomic single-stranded RNA directs the pathway toward a T=3 capsid. J Mol Biol. 2010;395:924-936.

21. Basore K, Kim AS, Nelson CA, et al. Cryo-EM structure of chikungunya virus in complex with the Mxra8 receptor. Cell. 2019;177:1725-1737 e16.

22. Basore K, Ma H, Kafai NM, et al. Structure of Venezuelan equine encephalitis virus in complex with the LDLRAD3 receptor. Nature. 2021;598:672-676.

23. Bauer PH, Cui C, Stehle T, Harrison SC, DeCaprio JA, Benjamin TL. Discrimination between sialic acid-containing receptors and pseudoreceptors regulates polyomavirus spread in the mouse. J Virol. 1999;73:5826-5832.

24. Belnap DM, Filman DJ, Trus BL, et al. Molecular tectonic model of virus structural transitions: the putative cell entry states of poliovirus. J Virol. 2000;74:1342-1354.

25. Belot L, Ouldali M, Roche S, Legrand P, Gaudin Y, Albertini AA. Crystal structure of Mokola virus glycoprotein in its post-fusion conformation. PLoS Pathog. 2020;16:e1008383.

26. Benson SD, Bamford JK, Bamford DH, Burnett RM. Viral evolution revealed by bacteriophages PRD1 and human adenovirus coat protein structures. Cell. 1999;98:825-833.

27. Benton DJ, Gamblin SJ, Rosenthal PB, Skehel JJ. Structural transitions in influenza haemagglutinin at membrane fusion pH. Nature. 2020;583:150-153.

28. Benton DJ, Nans A, Calder LJ, et al. Influenza hemagglutinin membrane anchor. Proc Natl Acad Sci U S A. 2018;115:10112-10117.

29. Blacklow SC, Lu M, Kim PS. A trimeric subdomain of the simian immunodeficiency virus envelope glycoprotein. Biochemistry. 1995;34:14955-14962.

30. Borodavka A, Ault J, Stockley PG, Tuma R. Evidence that avian reovirus sigmaNS is an RNA chaperone: implications for genome segment assortment. Nucleic Acids Res. 2015;43:7044-7057.

31. Borodavka A, Desselberger U, Patton JT. Genome packaging in multi-segmented dsRNA viruses: distinct mechanisms with similar outcomes. Curr Opin Virol. 2018;33:106-112.

32. Borodavka A, Dykeman EC, Schrimpf W, Lamb DC. Protein-mediated RNA folding governs sequence-specific interactions between rotavirus genome segments. Elife. 2017;6:e27453.

33. Bose S, Jardetzky TS, Lamb RA. Timing is everything: Fine-tuned molecular machines orchestrate paramyxovirus entry. Virology. 2015;479-480:518-531.

34. Bostina M, Bubeck D, Schwartz C, Nicastro D, Filman DJ, Hogle JM. Single particle cryoelectron tomography characterization of the structure and structural variability of poliovirus-receptor-membrane complex at 30 A resolution. J Struct Biol. 2007;160:200-210.

35. Brandenburg B, Lee LY, Lakadamyali M, Rust MJ, Zhuang X, Hogle JM. Imaging poliovirus entry in live cells. PLoS Biol. 2007;5:e183.

36. Bravo JPK, Bartnik K, Venditti L, et al. Structural basis of rotavirus RNA chaperone displacement and RNA annealing. Proc Natl Acad Sci U S A. 2021;118.

37. Briggs JA, Grunewald K, Glass B, Forster F, Krausslich HG, Fuller SD. The mechanism of HIV-1 core assembly: insights from three-dimensional reconstructions of authentic virions. Structure. 2006;14:15-20.

38. Brisco M, Hull R, Wilson TM. Swelling of isometric and of bacilliform plant virus nucleocapsids is required for virus-specific protein synthesis in vitro. Virology. 1986;148:210-217.

39. Brown RS, Anastasakis DG, Hafner M, Kielian M. Multiple capsid protein binding sites mediate selective packaging of the alphavirus genomic RNA. Nat Commun. 2020;11:4693.

40. Brown RS, Kim L, Kielian M. Specific recognition of a stem-loop RNA structure by the alphavirus capsid protein. Viruses. 2021;13.

41. Brown RS, Wan JJ, Kielian M. The alphavirus exit pathway: what we know and what we wish we knew. Viruses. 2018;10:89.

42. Bubeck D, Filman DJ, Cheng N, Steven AC, Hogle JM, Belnap DM. The structure of the poliovirus 135S cell entry intermediate at 10-angstrom resolution reveals the location of an externalized polypeptide that binds to membranes. J Virol. 2005;79:7745-7755.

43. Bubeck D, Filman DJ, Hogle JM. Cryo-electron microscopy reconstruction of a poliovirus-receptor-membrane complex. Nat Struct Mol Biol. 2005;12:615-618.

44. Bullough PA, Hughson FM, Skehel JJ, Wiley DC. The structure of influenza haemagglutinin at the pH of membrane fusion. Nature. 1994;371:37-43.

45. Burnie J, Guzzo C. The incorporation of host proteins into the external HIV-1 envelope. Viruses. 2019;11:85.

46. Butcher SJ, Dokland T, Ojala PM, Bamford DH, Fuller SD. Intermediates in the assembly pathway of the double-stranded RNA virus phi6. EMBO J. 1997;16:4477-4487.

47. Butler PJ, Finch JT, Zimmern D. Configuration of tobacco mosaic virus, RNA during virus assembly. Nature. 1977;265:217-219.

48. Button JM, Qazi SA, Wang JC, Mukhopadhyay S. Revisiting an old friend: new findings in alphavirus structure and assembly. Curr Opin Virol. 2020;45:25-33.

49. Calder LJ, Rosenthal PB. Cryomicroscopy provides structural snapshots of influenza virus membrane fusion. Nat Struct Mol Biol. 2016;23:853-858.

50. Calder LJ, Wasilewski S, Berriman JA, Rosenthal PB. Structural organization of a filamentous influenza A virus. Proc Natl Acad Sci U S A. 2010;107:10685-10690.

51. Carette JE, Raaben M, Wong AC, et al. Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. Nature 2011;477:340-343.

52. Carey J, Cameron V, de Haseth PL, Uhlenbeck OC. Sequence-specific interaction of R17 coat protein with its ribonucleic acid binding site. Biochemistry. 1983;22:2601-2610.

53. Carfi A, Willis SH, Whitbeck JC, et al. Herpes simplex virus glycoprotein D bound to the human receptor HveA. Mol Cell. 2001;8:169-179.

54. Casjens S, King J. Virus assembly. Annu Rev Biochem. 1975;44:555-611.

55. Casjens SR. The DNA-packaging nanomotor of tailed bacteriophages. Nat Rev Microbiol. 2011;9:647-657.

56. Caspar DL. Assembly and stability of the tobacco mosaic virus particle. Adv Protein Chem. 1963;18:37-121.

57. Caspar DL. Structure of bushy stunt virus. Nature. 1956;177:475-476.

58. Caspar DL. Movement and self-control in protein assemblies. Quasi-equivalence revisited. Biophys J. 1980;32:103-138.

59. Caspar DLD, Klug A. Physical principles in the construction of regular viruses. Cold Spr Harb Symp Quant Biol. 1962;27:1-24.

60. Catalano CE, Morais MC. Viral genome packaging machines: structure and enzymology. Enzymes. 2021;50:369-413.

61. Chan DC, Fass D, Berger JM, Kim PS. Core structure of gp41 from the HIV envelope glycoprotein. Cell. 1997;89:263-273.

62. Chandran K, Walker SB, Chen Y, et al. In vitro recoating of reovirus cores with baculovirus-expressed outer-capsid proteins mu1 and sigma3. J Virol. 1999;73:3941-3950.

63. Chang JT, Schmid MF, Rixon FJ, Chiu W. Electron cryotomography reveals the portal in the herpesvirus capsid. J Virol. 2007;81:2065-2068.

64. Chao LH, Klein DE, Schmidt AG, Pena JM, Harrison SC. Sequential conformational rearrangements in flavivirus membrane fusion. Elife. 2014;3:e04389.

65. Chen B. Molecular mechanism of HIV-1 entry. Trends Microbiol. 2019;27:878-891.

66. Chen BJ, Lamb RA. Mechanisms for enveloped virus budding: can some viruses do without an ESCRT? Virology. 2008;372:221-232.

67. Chen J, Kovacs JM, Peng H, et al. HIV-1 ENVELOPE. Effect of the cytoplasmic domain on antigenic characteristics of HIV-1 envelope glycoprotein. Science. 2015;349:191-195.

68. Chen J, Skehel JJ, Wiley DC. N- and C-terminal residues combine in the fusion-pH influenza hemagglutinin HA(2) subunit to form an N cap that terminates the triple-stranded coiled coil. Proc Natl Acad Sci U S A. 1999;96:8967-8972.

69. Chen L, Gorman JJ, McKimm-Breschkin J, et al. The structure of the fusion glycoprotein of Newcastle disease virus suggests a novel paradigm for the molecular mechanism of membrane fusion. Structure. 2001;9:255-266.

70. Chen L, Wang M, Zhu D, et al. Implication for alphavirus host-cell entry and assembly indicated by a 3.5A resolution cryo-EM structure. Nat Commun. 2018;9:5326.

71. Chen R, Neill JD, Estes MK, Prasad BV. X-ray structure of a native calicivirus: structural insights into antigenic diversity and host specificity. Proc Natl Acad Sci U S A. 2006;103:8048-8053.

72. Chen ZG, Stauffacher C, Li T, et al. Protein-RNA interactions in an icosahedral virus at 3.0 A resolution. Science. 1989;245:154-159.

73. Cheng RH, Kuhn RJ, Olson NH, et al. Nucleocapsid and glycoprotein organization in an enveloped virus. Cell. 1995;80:621-630.

74. Chmielewski D, Schmid MF, Simmons G, Jin J, Chiu W. Chikungunya virus assembly and budding visualized in situ using cryogenic electron tomography. Nat Microbiol. 2022;7(8):1270-1279. doi:10.1038/s41564-022-01164-2

75. Chou YY, Vafabakhsh R, Doganay S, Gao Q, Ha T, Palese P. One influenza virus particle packages eight unique viral RNAs as shown by FISH analysis. Proc Natl Acad Sci U S A. 2012;109:9101-9106.

76. Chow M, Newman JF, Filman D, Hogle JM, Rowlands JD, Brown F. Myristoylation of picornavirus capsid protein VP4 and its structural significance. Nature. 1987;327:482-486.

77. Chowdary TK, Cairns TM, Atanasiu D, Cohen GH, Eisenberg RJ, Heldwein EE. Crystal structure of the conserved herpesvirus fusion regulator complex gH-gL. Nat Struct Mol Biol. 2010;17:882-888.

78. Cockburn JJ, Abrescia NG, Grimes JM, et al. Membrane structure and interactions with protein and DNA in bacteriophage PRD1. Nature. 2004;432:122-125.

79. Colman PM. Virus versus antibody. Structure. 1997;5:591-593.

80. Conley MJ, McElwee M, Azmi L, et al. Calicivirus VP2 forms a portal-like assembly following receptor engagement. Nature. 2019;565:377-381.

81. Connolly SA, Leser GP, Yin HS, Jardetzky TS, Lamb RA. Refolding of a paramyxovirus F protein from prefusion to postfusion conformations observed by liposome binding and electron microscopy. Proc Natl Acad Sci U S A. 2006;103:17903-17908.

82. Conway JF, Wikoff WR, Cheng N, et al. Virus maturation involving large subunit rotations and local refolding. Science. 2001;292:744-748.

83. Coulibaly F, Chevalier C, Gutsche I, et al. The birnavirus crystal structure reveals structural relationships among icosahedral viruses. Cell. 2005;120:761-772.

84. Crawford SE, Mukherjee SK, Estes MK, et al. Trypsin cleavage stabilizes the rotavirus VP4 spike. J Virol. 2001;75:6052-6061.

85. Crick FHC, Watson JD. Structure of small viruses. Nature (London). 1956;177:473-375.

86. Crowther RA, Amos LA. Three-dimensional image reconstructions of some small spherical viruses. Cold Spring Harb Symp Quant Biol. 1972;36:489-494.

87. Crowther RA, DeRosier DJ, Klug A. The reconstruction of a three-dimensional structure from projections and its application to electron microscopy. Proc R Soc Lond A. 1970;317:319-340.

88. Crowther RA, Klug A. Structural analysis of macromolecular assemblies by image reconstruction from electron micrographs. Annu Rev Biochem. 1975;44:161-182.

89. Cureton DK, Harbison CE, Cocucci E, Parrish CR, Kirchhausen T. Limited transferrin receptor clustering allows rapid diffusion of canine parvovirus into clathrin endocytic structures. J Virol. 2012;86:5330-5340.

90. Cyrklaff M, Risco C, Fernandez JJ, et al. Cryo-electron tomography of vaccinia virus. Proc Natl Acad Sci U S A. 2005;102:2772-2777.

91. Dai X, Li Z, Lai M, et al. In situ structures of the genome and genome-delivery apparatus in a single-stranded RNA virus. Nature. 2017;541:112-116.

92. Dai X, Wu L, Sun R, Zhou ZH. Atomic structures of minor proteins VI and VII in human adenovirus. J Virol. 2017;91:e00850-17.

93. Danieli T, Pelletier SL, Henis YI, White JM. Membrane fusion mediated by the influenza virus hemagglutinin requires the concerted action of at least three hemagglutinin trimers. J Cell Biol. 1996;133:559-569.

94. Daniels RS, Downie JC, Hay AJ, et al. Fusion mutants of the influenza virus hemagglutinin glycoprotein. Cell. 1985;40:431-439.

95. Danthi P, Tosteson M, Li QH, Chow M. Genome delivery and ion channel properties are altered in VP4 mutants of poliovirus. J Virol. 2003;77:5266-5274.

96. De Guzman RN, Wu ZR, Stalling CC, Papparlardo L, Borer PN, Summers MF. Structure of the HIV-1 nucleocapsid protein bound to the SL3 psi-RNA recognition element. Science. 1998;279:384-388.

97. de Haas F, Paatero AO, Mindich L, Bamford DH, Fuller SD. A symmetry mismatch at the site of RNA packaging in the polymerase complex of dsRNA bacteriophage phi6. J Mol Biol. 1999;294:357-372.

98. Dedeo CL, Cingolani G, Teschke CM. Portal protein: the orchestrator of capsid assembly for the dsDNA tailed bacteriophages and herpesviruses. Annu Rev Virol. 2019;6:141-160.

99. de Haan CA, Vennema H, Rottier PJ. Assembly of the coronavirus envelope: homotypic interactions between the M proteins. J Virol. 2000;74:4967-4978.

100. de Rosny E, Vassell R, Jiang S, Kunert R, Weiss CD. Binding of the 2F5 monoclonal antibody to native and fusion-intermediate forms of human immunodeficiency virus type 1 gp41: implications for fusion-inducing conformational changes. J Virol. 2004;78:2627-2631.

101. DeRosier DJ, Klug A. Reconstruction of three dimensional structures from electron micrographs. Nature. 1968;217:130-134.

102. Dessau M, Modis Y. Crystal structure of glycoprotein C from Rift Valley fever virus. Proc Natl Acad Sci U S A. 2013;110:1696-1701.

103. Dev J, Park D, Fu Q, et al. Structural basis for membrane anchoring of HIV-1 envelope spike. Science. 2016;353:172-175.

104. Dietrich MH, Ogden KM, Long JM, et al. Structural and functional features of the Reovirus sigma1 tail. J Virol. 2018;92:e00336-18.

105. Ding P, Kharytonchyk S, Waller A, et al. Identification of the initial nucleocapsid recognition element in the HIV-1 RNA packaging signal. Proc Natl Acad Sci U S A. 2020;117:17737-17746.

106. Dormitzer PR, Nason EB, Prasad BV, Harrison SC. Structural rearrangements in the membrane penetration protein of a non-enveloped virus. Nature. 2004;430:1053-1058.

107. Draganova EB, Valentin J, Heldwein EE. The ins and outs of herpesviral capsids: divergent structures and assembly mechanisms across the three subfamilies. Viruses. 2021; 13:1913.

108. Dreyfus C, Laursen NS, Kwaks T, et al. Highly conserved protective epitopes on influenza B viruses. Science. 2012;337:1343-1348.

109. Dryden KA, Wang G, Yeager M, et al. Early steps in reovirus infection are associated with dramatic changes in supramolecular structure and protein conformation: analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction. J Cell Biol. 1993;122:1023-1041.

110. D'Souza V, Summers MF. How retroviruses select their genomes. Nat Rev Microbiol. 2005;3:643-655.

111. Duda RL, Hempel J, Michel H, Shabanowitz J, Hunt D, Hendrix RW. Structural transitions during bacteriophage HK97 head assembly. J Mol Biol. 1995;247:618-635.

112. Duquerroy S, Da Costa B, Henry C, et al. The picobirnavirus crystal structure provides functional insights into virion assembly and cell entry. EMBO J. 2009;28:1655-1665.

113. Durrer P, Gaudin Y, Ruigrok RW, Graf R, Brunner J. Photolabeling identifies a putative fusion domain in the envelope glycoprotein of rabies and vesicular stomatitis viruses. J Biol Chem. 1995;270:17575-17581.

114. Earnshaw W, Casjens S, Harrison SC. Assembly of the head of bacteriophage P22: x-ray diffraction from heads, proheads and related structures. J Mol Biol. 1976;104:387-410.

115. Earnshaw WC, Harrison SC. DNA arrangement in isometric phage heads. Nature. 1977;268:598-602.

116. Earnshaw WC, King J, Harrison SC, Eiserling FA. The structural organization of DNA packaged within the heads of T4 wild-type, isometric and giant bacteriophages. Cell. 1978;14:559-568.

117. Eisfeld AJ, Neumann G, Kawaoka Y. At the centre: influenza A virus ribonucleoproteins. Nat Rev Microbiol. 2015;13:28-41.

118. Emini EA, Ostapchuk P, Wimmer E. Bivalent attachment of antibody onto poliovirus leads to conformational alteration and neutralization. J Virol. 1983;48:547-550.

119. Feng Z, Hensley L, McKnight KL, et al. A pathogenic picornavirus acquires an envelope by hijacking cellular membranes. Nature. 2013;496:367-371.

120. Fisher AJ, Johnson JE. Ordered duplex RNA controls capsid architecture in an icosahedral animal virus. Nature. 1993;361:176-179.

121. FitzGerald DJ, Padmanabhan R, Pastan I, Willingham MC. Adenovirus-induced release of epidermal growth factor and pseudomonas toxin into the cytosol of KB cells during receptor-mediated endocytosis. Cell. 1983;32:607-617.

122. Floyd DL, Ragains JR, Skehel JJ, Harrison SC, van Oijen AM. Single-particle kinetics of influenza virus membrane fusion. Proc Natl Acad Sci U S A. 2008;105:15382-15387.

123. Fokine A, Rossmann MG. Molecular architecture of tailed double-stranded DNA phages. Bacteriophage. 2014;4:e28281.

124. Freedman H, Logan MR, Law JL, Houghton M. Structure and function of the hepatitis C virus envelope glycoproteins E1 and E2: antiviral and vaccine targets. ACS Infect Dis. 2016;2:749-762.

125. Fricks CE, Hogle JM. Cell-induced conformational change in poliovirus: externalization of the amino terminus of VP1 is responsible for liposome binding. J Virol. 1990;64:1934-1945.

126. Frolova E, Frolov I, Schlesinger S. Packaging signals in alphaviruses. J Virol. 1997;71:248-258.

127. Fu Q, Shaik MM, Cai Y, et al. Structure of the membrane proximal external region of HIV-1 envelope glycoprotein. Proc Natl Acad Sci U S A. 2018;115:E8892-E8899.

128. Furciniti PS, Van Oostrum J, Burnett RM. Adenovirus polypeptide IX revealed as capsid cement by difference images from electron microscopy and crystallography. EMBO J. 1989;8:3563-3570.

129. Furuta RA, Wild CT, Weng Y, Weiss CD. Capture of an early fusion-active conformation of HIV-1 gp41. Nat Struct Biol. 1998;5:276-279.

130. Gamblin SJ, Skehel JJ. Influenza hemagglutinin and neuraminidase membrane glycoproteins. J Biol Chem. 2010;285:28403-28409.

131. Ganser BK, Li S, Klishko VY, Finch JT, Sundquist WI. Assembly and analysis of conical models for the HIV-1 core. Science. 1999;283:80-83.

132. Ganser-Pornillos BK, Yeager M, Sundquist WI. The structural biology of HIV assembly. Curr Opin Struct Biol. 2008;18:203-217.

133. Garbutt M, Liebscher R, Wahl-Jensen V, et al. Properties of replication-competent vesicular stomatitis virus vectors expressing glycoproteins of filoviruses and arenaviruses. J Virol. 2004;78:5458-5465.

134. Garrus JE, von Schwedler UK, Pornillos OW, et al. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell. 2001;107:55-65.

135. Gilman MSA, Liu C, Fung A, et al. Structure of the respiratory syncytial virus polymerase complex. Cell. 2019;179:193-204 e14.

136. Golden JS, Harrison SC. Proteolytic dissection of turnip crinkle virus subunit in solution. Biochemistry. 1982;21:3862-3866.

137. Gomez Yafal A, Kaplan G, Racaniello VR, Hogle JM. Characterization of poliovirus conformational alteration mediated by soluble cell receptors. Virology. 1993;197:501-505.

138. Goto H, Muramoto Y, Noda T, Kawaoka Y. The genome-packaging signal of the influenza A virus genome comprises a genome incorporation signal and a genome-bundling signal. J Virol. 2013;87:11316-11322.

139. Gowen B, Bamford JK, Bamford DH, Fuller SD. The tailless icosahedral membrane virus PRD1 localizes the proteins involved in genome packaging and injection at a unique vertex. J Virol. 2003;77:7863-7871.

140. Gralla J, DeLisi C. mRNA is expected to form stable secondary structures. Nature. 1974;248:330-332.

141. Greber UF, Willetts M, Webster P, Helenius A. Stepwise dismantling of adenovirus 2 during entry into cells. Cell. 1993;75:477-486.

142. Green TJ, Zhang X, Wertz GW, Luo M. Structure of the vesicular stomatitis virus nucleoprotein-RNA complex. Science. 2006;313:357-360.

143. Grigorieff N, Harrison SC. Near-atomic resolution reconstructions of icosahedral viruses from electron cryo-microscopy. Curr Opin Struct Biol. 2011;21:265-273.

144. Grimes J, Basak AK, Roy P, Stuart D. The crystal structure of bluetongue virus VP7. Nature. 1995;373:167-170.

145. Grimes JM, Burroughs JM, Gouet P, et al. The atomic structure of the bluetongue virus core. Nature. 1998;395:470-478.

146. Grimes S, Jardine PJ, Anderson D. Bacteriophage phi 29 DNA packaging. Adv Virus Res. 2002;58:255-294.

Grunewald K, Cyrklaff M. Structure of complex viruses and virus-infected cells by electron cryo tomography. *Curr Opin Microbiol*. 2006;9:437-442.

147. Grunewald K, Desai P, Winkler DC, et al. Three-dimensional structure of herpes simplex virus from cryo-electron tomography. Science. 2003;302:1396-1398.

148. Guardado-Calvo P, Atkovska K, Jeffers SA, et al. A glycerophospholipid-specific pocket in the RVFV class II fusion protein drives target membrane insertion. Science. 2017;358: 663-667.

149. Guardado-Calvo P, Bignon EA, Stettner E, et al. Mechanistic insight into Bunyavirus-induced membrane fusion from structure-function analyses of the Hantavirus envelope glycoprotein Gc. PLoS Pathog. 2016;12:e1005813.

150. Guardado-Calvo P, Rey FA. The surface glycoproteins of hantaviruses. Curr Opin Virol. 2021;50:87-94.

151. Guardado-Calvo P, Rey FA. The viral class II Membrane fusion machinery: divergent evolution from an ancestral heterodimer. Viruses. 2021;13:2368.

152. Guogas LM, Filman DJ, Hogle JM, Gehrke L. Cofolding organizes alfalfa mosaic virus RNA and coat protein for replication. Science. 2004;306:2108-2111.

153. Guttman N, Baltimore D. A plasma membrane component able to bind and alter virions of poliovirus type 1: studies on cell-free alteration using a simplified assay. Virology. 1977; 82:25-36.

154. Guzzo C, Ichikawa D, Park C, et al. Virion incorporation of integrin alpha4beta7 facilitates HIV-1 infection and intestinal homing. Sci Immunol. 2017;2.

155. Hall L, Rueckert RR. Infection of mouse fibroblasts by cardioviruses: premature uncoating and its prevention by elevated pH and magnesium chloride. Virology. 1971;43:152-165.

156. Halldorsson S, Behrens AJ, Harlos K, et al. Structure of a phleboviral envelope glycoprotein reveals a consolidated model of membrane fusion. Proc Natl Acad Sci U S A. 2016;113:7154-7159.

157. Halldorsson S, Li S, Li M, Harlos K, Bowden TA, Huiskonen JT. Shielding and activation of a viral membrane fusion protein. Nat Commun. 2018;9:349.

158. Hannah BP, Heldwein EE, Bender FC, Cohen GH, Eisenberg RJ. Mutational evidence of internal fusion loops in herpes simplex virus glycoprotein B. J Virol. 2007;81:4858-4865.

159. Harrison SC. Protein interfaces and intersubunit bonding. The case of tomato bushy stunt virus. Biophys J. 1980;32:139-153.

160. Harrison SC. Packaging of DNA into bacteriophage heads: a model. J Mol Biol. 1983;171:577-580.

161. Harrison SC. Viral membrane fusion. Nat Struct Mol Biol. 2008;15:690-698.

162. Harrison SC. Viral membrane fusion. Virology. 2015;479-480:498-507.

163. Harrison SC, Olson A, Schutt CE, Winkler FK, Bricogne G. Tomato bushy stunt virus at 2.9 Å resolution. Nature (London). 1978;276:368-373.

164. He Y, Vassell R, Zaitseva M, et al. Peptides trap the human immunodeficiency virus type 1 envelope glycoprotein fusion intermediate at two sites. J Virol. 2003;77:1666-1671.

165. Heldwein EE, Lou H, Bender FC, Cohen GH, Eisenberg RJ, Harrison SC. Crystal structure of glycoprotein B from herpes simplex virus 1. Science. 2006;313:217-220.

166. Henderson R. The potential and limitations of neutrons, electrons and x-rays for atomic resolution microscopy of unstained biological molecules. Q Rev Biophys. 1995;28:171-193.

167. Henderson R. From electron crystallography to single particle cryoEM. Angew Chem Int Ed Engl. 2018;57:10804-10825.

168. Hendrix RW, Johnson JE. Bacteriophage HK97 capsid assembly and maturation. Adv Exp Med Biol. 2012;726:351-363.

169. Hernandez JM, Podbilewicz B. The hallmarks of cell-cell fusion. Development. 2017;144:4481-4495.

170. Herrler G, Rott R, Klenk HD, Muller HP, Shukla AK, Schauer R. The receptor-destroying enzyme of influenza C virus is neuraminate-O-acetylesterase. EMBO J. 1985;4:1503-1506.

171. Herrmann T, Torres R, Salgado EN, et al. Functional refolding of the penetration protein on a non-enveloped virus. Nature. 2021;590:666-670.

172. Hillen HS, Kokic G, Farnung L, Dienemann C, Tegunov D, Cramer P. Structure of replicating SARS-CoV-2 polymerase. Nature. 2020;584:154-156.

173. Hogle JM. Poliovirus cell entry: common structural themes in viral cell entry pathways. Annu Rev Microbiol. 2002;56:677-702.

174. Hogle JM, Chow M, Filman DJ. Three-dimensional structure of poliovirus at 2.9 Å resolution. Science. 1985;229:1358-1365.

175. Hooper JW, Fields BN. Role of the mu 1 protein in reovirus stability and capacity to cause chromium release from host cells. J Virol. 1996;70:459-467.

176. Horwitz JA, Jenni S, Harrison SC, Whelan SPJ. Structure of a rabies virus polymerase complex from electron cryo-microscopy. Proc Natl Acad Sci U S A. 2020;117:2099-2107.

177. Hsieh CL, Goldsmith JA, Schaub JM, et al. Structure-based design of prefusion-stabilized SARS-CoV-2 spikes. Science. 2020;369:1501-1505.

178. Hsieh CL, Rush SA, Palomo C, et al. Structure-based design of prefusion-stabilized human metapneumovirus fusion proteins. Nat Commun. 2022;13:1299.

179. Hu L, Chow DC, Patton JT, Palzkill T, Estes MK, Prasad BV. Crystallographic Analysis of Rotavirus NSP2-RNA Complex Reveals Specific Recognition of 5′ GG Sequence for RTPase Activity. J Virol. 2012;86:10547-10557.

180. Huang RK, Khayat R, Lee KK, et al. The Prohead-I structure of bacteriophage HK97: implications for scaffold-mediated control of particle assembly and maturation. J Mol Biol. 2011;408:541-554.

181. Huiskonen JT, de Haas F, Bubeck D, Bamford DH, Fuller SD, Butcher SJ. Structure of the bacteriophage phi6 nucleocapsid suggests a mechanism for sequential RNA packaging. Structure. 2006;14:1039-1048.

182. Huiskonen JT, Overby AK, Weber F, Grunewald K. Electron cryo-microscopy and single-particle averaging of Rift Valley fever virus: evidence for GN-GC glycoprotein heterodimers. J Virol. 2009;83:3762-3769.

183. Hulswit RJG, Paesen GC, Bowden TA, Shi X. Recent advances in bunyavirus glycoprotein research: precursor processing, receptor binding and structure. Viruses. 2021; 13:353.

184. Ilca SL, Sun X, El Omari K, et al. Multiple liquid crystalline geometries of highly compacted nucleic acid in a dsRNA virus. Nature. 2019;570:252-256.

185. Ivanova PT, Myers DS, Milne SB, McClaren JL, Thomas PG, Brown HA. Lipid composition of viral envelope of three strains of influenza virus—not all viruses are created equal. ACS Infect Dis. 2015;1:399-452.

186. Ivanovic T, Agosto MA, Zhang L, Chandran K, Harrison SC, Nibert ML. Peptides released from reovirus outer capsid form membrane pores that recruit virus particles. EMBO J. 2008;27:1289-1298.

187. Ivanovic T, Choi JL, Whelan SP, van Oijen AM, Harrison SC. Influenza-virus membrane fusion by cooperative fold-back of stochastically induced hemagglutinin intermediates. Elife. 2013;2:e00333.

188. Ivanovic T, Harrison SC. Distinct functional determinants of influenza hemagglutinin-mediated membrane fusion. Elife. 2015;4:e11009.

189. Iyer LM, Makarova KS, Koonin EV, Aravind L. Comparative genomics of the FtsK-HerA superfamily of pumping ATPases: implications for the origins of chromosome segregation, cell division and viral capsid packaging. Nucleic Acids Res. 2004;32:5260-5279.

190. Jaalinoja HT, Huiskonen JT, Butcher SJ. Electron cryomicroscopy comparison of the architectures of the enveloped bacteriophages phi6 and phi8. Structure. 2007;15:157-167.

191. Jackson CB, Farzan M, Chen B, Choe H. Mechanisms of SARS-CoV-2 entry into cells. Nat Rev Mol Cell Biol. 2022;23:3-20.

192. Jayaram H, Taraporewala Z, Patton JT, Prasad BV. Rotavirus protein involved in genome replication and packaging exhibits a HIT-like fold. Nature. 2002;417:311-315.

193. Jenni S, Bloyet LM, Diaz-Avalos R, et al. Structure of the vesicular stomatitis virus L protein in complex with its phosphoprotein cofactor. Cell Rep. 2020;30:53-60 e5.

194. Jenni S, Horwitz JA, Bloyet LM, Whelan SPJ, Harrison SC. Visualizing molecular interactions that determine assembly of a bullet-shaped vesicular stomatitis virus particle. Nat Commun. 2022;13(1):4802.

195. Jenni S, Salgado EN, Herrmann T, et al. In situ structure of rotavirus VP1 RNA-dependent RNA polymerase. J Mol Biol. 2019;431:3124-3138.

196. Jiang W, Li Z, Zhang Z, Baker ML, Prevelige PE Jr, Chiu W. Coat protein fold and maturation transition of bacteriophage P22 seen at subnanometer resolutions. Nat Struct Biol. 2003;10:131-135.

197. Jiang X, Jayaram H, Kumar M, Ludtke SJ, Estes MK, Prasad BV. Cryoelectron microscopy structures of rotavirus NSP2-NSP5 and NSP2-RNA complexes: implications for genome replication. J Virol. 2006;80:10829-10835.

198. Joglekar AV, Sandoval S. Pseudotyped Lentiviral Vectors: One Vector, Many Guises. Hum Gene Ther Methods. 2017;28:291-301.

199. Johnson JE. Virus particle maturation: insights into elegantly programmed nanomachines. Curr Opin Struct Biol. 2010;20:210-216.

200. Julien JP, Cupo A, Sok D, et al. Crystal structure of a soluble cleaved HIV-1 envelope trimer. Science. 2013;342:1477-1483.

201. Jung J, Grant T, Thomas DR, Diehnelt CW, Grigorieff N, Joshua-Tor L. High-resolution cryo-EM structures of outbreak strain human norovirus shells reveal size variations. Proc Natl Acad Sci U S A. 2019;116:12828-12832.

202. Kadlec J, Loureiro S, Abrescia NG, Stuart DI, Jones IM. The postfusion structure of baculovirus gp64 supports a unified view of viral fusion machines. Nat Struct Mol Biol. 2008;15:1024-1030.

203. Kang YK, Andjelic S, Binley JM, et al. Structural and immunogenicity studies of a cleaved, stabilized envelope trimer derived from subtype A HIV-1. Vaccine. 2009;27:5120-5132.

204. Keane SC, Heng X, Lu K, et al. RNA structure. Structure of the HIV-1 RNA packaging signal. Science. 2015;348:917-921.

205. Kerviel A, Zhang M, Altan-Bonnet N. A new infectious unit: extracellular vesicles carrying virus populations. Annu Rev Cell Dev Biol. 2021;37:171-197.

206. Kim DY, Firth AE, Atasheva S, Frolova EI, Frolov I. Conservation of a packaging signal and the viral genome RNA packaging mechanism in alphavirus evolution. J Virol. 2011;85:8022-8036.

207. Kim IS, Jenni S, Stanifer ML, et al. Mechanism of membrane fusion induced by vesicular stomatitis virus G protein. Proc Natl Acad Sci U S A. 2017;114:E28-E36.

208. Kim IS, Trask SD, Babyonyshev M, Dormitzer PR, Harrison SC. Effect of mutations in VP5 hydrophobic loops on rotavirus cell entry. J Virol. 2010;84:6200-6207.

209. Klenk HD, Choppin PW. Lipids of plasma membranes of monkey and hamster kidney cells and of parainfluenza virions grown in these cells. Virology. 1969;38:255-268.

210. Klug A, Finch JT. Structure of viruses of the papilloma-polyoma type. I. Human Wart virus. J Mol Biol. 1965;11:403-423.

211. Koning RI, Gomez-Blanco J, Akopjana I, et al. Asymmetric cryo-EM reconstruction of phage MS2 reveals genome structure in situ. Nat Commun. 2016;7:12524.

212. Kornfeind EM, Visalli RJ. Human herpesvirus portal proteins: structure, function, and antiviral prospects. Rev Med Virol. 2018;28:e1972.

213. Krummenacher C, Supekar VM, Whitbeck JC, et al. Structure of unliganded HSV gD reveals a mechanism for receptor-mediated activation of virus entry. EMBO J. 2005;24:4144-4153.

214. Kuhlbrandt W. Biochemistry. The resolution revolution. Science. 2014;343:1443-1444.

215. Kuhn RJ, Zhang W, Rossmann MG, et al. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell. 2002;108:717-725.

216. Kumar A, Hossain RA, Yost SA, et al. Structural insights into hepatitis C virus receptor binding and entry. Nature. 2021;598:521-525.

217. Kumar M, Jayaram H, Vasquez-Del Carpio R, et al. Crystallographic and biochemical analysis of rotavirus NSP2 with nucleotides reveals a nucleoside diphosphate kinase-like activity. J Virol. 2007;81:12272-12284.

218. Kundhavai Natchiar S, Venkataraman S, Mullen TM, Nemerow GR, Reddy VS. Revised crystal structure of human adenovirus reveals the limits on protein IX quasi-equivalence and on analyzing large macromolecular complexes. J Mol Biol. 2018;430:4132-4141.

219. Kuzmin PI, Zimmerberg J, Chizmadzhev YA, Cohen FS. A quantitative model for membrane fusion based on low-energy intermediates. Proc Natl Acad Sci U S A. 2001;98: 7235-7240.

220. Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature. 1998;393:648-659.

221. Lamb RA, Jardetzky TS. Structural basis of viral invasion: lessons from paramyxovirus F. Curr Opin Struct Biol. 2007;17:427-436.

222. Lata R, Conway JF, Cheng N, et al. Maturation dynamics of a viral capsid: visualization of transitional intermediate states. Cell. 2000;100:253-263.

223. Laver WG, Wrigley NG, Pereira HG. Removal of pentons from particles of adenovirus type 2. Virology. 1969;39:599-604.

224. Lee JH, Ozorowski G, Ward AB. Cryo-EM structure of a native, fully glycosylated, cleaved HIV-1 envelope trimer. Science. 2016;351:1043-1048.

224a. Leiman PG, Arisaki F, van Raaij MJ, et al. Morphogenesis of the T4 head and tail fibers. *Virol J*. 2010;7:355.

225. Leis A, Rockel B, Andrees L, Baumeister W. Visualizing cells at the nanoscale. Trends Biochem Sci. 2009;34:60-70.

226. Li F, Li W, Farzan M, Harrison SC. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. Science. 2005;309:1864-1868.

227. Li L, Lok SM, Yu IM, et al. The flavivirus precursor membrane-envelope protein complex: structure and maturation. Science. 2008;319:1830-1834.

228. Li S, Rissanen I, Zeltina A, et al. A molecular-level account of the antigenic hantaviral surface. Cell Rep. 2016;15:959-967.

229. Li T, Li Z, Deans EE, et al. The shape of pleomorphic virions determines resistance to cell-entry pressure. Nat Microbiol. 2021;6:617-629.

230. Li X, Wang L, Wang X, et al. Structure of RdRps within a transcribing dsRNA virus provides insights into the mechanisms of RNA synthesis. J Mol Biol. 2020;432:358-366.

231. Liang B, Li Z, Jenni S, et al. Structure of the L protein of vesicular stomatitis virus from electron cryomicroscopy. Cell. 2015;162:314-327.

232. Liddington RC, Yan Y, Zhao HC, Sahli R, Benjamin TL, Harrison SC. Structure of simian virus 40 at 3.8 Å resolution. Nature. 1991;354:278-284.

233. Liemann S, Chandran K, Baker TS, Nibert ML, Harrison SC. Structure of the reovirus membrane-penetration protein, Mu1, in a complex with is protector protein, Sigma3. Cell. 2002;108:283-295.

234. Liljas L, Unge T, Jones TA, et al. Structure of satellite tobacco necrosis virus at 3.0A resolution. J Mol Biol. 1982;159:93-108.

235. Liu H, Cheng L. Cryo-EM shows the polymerase structures and a nonspooled genome within a dsRNA virus. Science. 2015;349:1347-1350.

236. Liu H, Jin L, Koh SB, et al. Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. Science. 2010;329:1038-1043.

237. Liu J, Bartesaghi A, Borgnia MJ, Sapiro G, Subramaniam S. Molecular architecture of native HIV-1 gp120 trimers. Nature. 2008;455:109-113.

238. Liu Q, Acharya P, Dolan MA, et al. Quaternary contact in the initial interaction of CD4 with the HIV-1 envelope trimer. Nat Struct Mol Biol.

2017;24:370-378.

239. Liu ST, Sharon-Friling R, Ivanova P, et al. Synaptic vesicle-like lipidome of human cytomegalovirus virions reveals a role for SNARE machinery in virion egress. Proc Natl Acad Sci U S A. 2011;108:12869-12874.

240. Liu Y, Heim KP, Che Y, et al. Prefusion structure of human cytomegalovirus glycoprotein B and structural basis for membrane fusion. Sci Adv. 2021;7:eabf3178.

241. Liu YT, Jih J, Dai X, Bi GQ, Zhou ZH. Cryo-EM structures of herpes simplex virus type 1 portal vertex and packaged genome. Nature. 2019;570:257-261.

242. Lorieau JL, Louis JM, Bax A. The complete influenza hemagglutinin fusion domain adopts a tight helical hairpin arrangement at the lipid:water interface. Proc Natl Acad Sci U S A. 2010;107:11341-11346.

243. Lorieau JL, Louis JM, Bax A. Helical hairpin structure of influenza hemagglutinin fusion peptide stabilized by charge-dipole interactions between the N-terminal amino group and the second helix. J Am Chem Soc. 2011;133:2824-2827.

244. Lourenco S, Roy P. In vitro reconstitution of Bluetongue virus infectious cores. Proc Natl Acad Sci U S A. 2011;108:13746-13751.

245. Lu K, Heng X, Garyu L, et al. NMR detection of structures in the HIV-1 5′-leader RNA that regulate genome packaging. Science. 2011;334:242-245.

246. Lu M, Blacklow SC, Kim PS. A trimeric structural domain of the HIV-1 transmembrane glycoprotein. Nat Struct Biol. 1995;2:1075-1082.

247. Lu X, McDonald SM, Tortorici MA, et al. Mechanism for coordinated RNA packaging and genome replication by rotavirus polymerase VP1. Structure. 2008;16:1678-1688.

248. Lyumkis D, Julien JP, de Val N, et al. Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 envelope trimer. Science. 2013;342:1484-1490.

249. Markin VS, Kozlov MM, Borovjagin VL. On the theory of membrane fusion. The stalk mechanism. Gen Physiol Biophys. 1984;3:361-377.

250. Markosyan RM, Cohen FS, Melikyan GB. HIV-1 envelope proteins complete their folding into six-helix bundles immediately after fusion pore formation. Mol Biol Cell. 2003;14:926-938.

251. Mathieu M, Petitpas I, Navaza J, et al. Atomic structure of the major capsid protein of rotavirus: implications for the architecture of the virion. Embo J. 2001;20:1485-1497.

252. Matsuura H, Kirschner AN, Longnecker R, Jardetzky TS. Crystal structure of the Epstein-Barr virus (EBV) glycoprotein H/glycoprotein L (gH/gL) complex. Proc Natl Acad Sci U S A. 2010;107:22641-22646.

253. Mattei S, Glass B, Hagen WJ, Krausslich HG, Briggs JA. The structure and flexibility of conical HIV-1 capsids determined within intact virions. Science. 2016;354:1434-1437.

254. McClain B, Settembre E, Temple BR, Bellamy AR, Harrison SC. X-ray crystal structure of the rotavirus inner capsid particle at 3.8 A resolution. J Mol Biol. 2010;397:587-599.

255. McKenna R, Xia D, Willingman P, et al. Atomic structure of single-stranded DNA bacteriophage ΦX174 and its functional implications. Nature. 1992;355:137-143.

256. McLellan JS, Chen M, Leung S, et al. Structure of RSV fusion glycoprotein trimer bound to a prefusion-specific neutralizing antibody. Science. 2013;340:1113-1117.

257. Merchant M, Mata CP, Liu Y, Zhai H, Protasio AV, Modis Y. A bioactive phlebovirus-like envelope protein in a hookworm endogenous virus. Sci Adv. 2022;8:eabj6894.

258. Metsikko K, Garoff H. Role of heterologous and homologous glycoproteins in phenotypic mixing between Sendai virus and vesicular stomatitis virus. J Virol. 1989;63:5111-5118.

259. Metz SW, Thomas A, White L, et al. Dengue virus-like particles mimic the antigenic properties of the infectious dengue virus envelope. Virol J. 2018;15:60.

260. Mietzsch M, Penzes JJ, Agbandje-McKenna M. Twenty-five years of structural parvovirology. Viruses. 2019;11:362.

261. Mindich L. Packaging, replication and recombination of the segmented genome of bacteriophage Phi6 and its relatives. Virus Res. 2004;101:83-92.

262. Modis Y, Ogata S, Clements D, Harrison SC. Structure of the dengue virus envelope protein after membrane fusion. Nature. 2004;427:313-319.

263. Moeller A, Kirchdoerfer RN, Potter CS, Carragher B, Wilson IA. Organization of the influenza virus replication machinery. Science. 2012;338:1631-1634.

264. Moffitt JR, Chemla YR, Aathavan K, et al. Intersubunit coordination in a homomeric ring ATPase. Nature. 2009;457:446-450.

265. Moody MF. Structure of the sheath of bacteriophage T4. II. Rearrangement of the sheath subunits during contraction. J Mol Biol. 1967;25:201-208.

266. Moody MF. Geometry of phage head construction. J Mol Biol. 1999;293:401-433.

267. Moore JP, McKeating JA, Weiss RA, Sattentau QJ. Dissociation of gp120 from HIV-1 virions induced by soluble CD4. Science. 1990;250:1139-1142.

268. Moscufo N, Yafal AG, Rogove A, Hogle J, Chow M. A mutation in VP4 defines a new step in the late stages of cell entry by poliovirus. J Virol. 1993;67:5075-5078.

269. Moyer CL, Wiethoff CM, Maier O, Smith JG, Nemerow GR. Functional genetic and biophysical analyses of membrane disruption by human adenovirus. J Virol. 2011;85:2631-2641.

270. Mukhopadhyay S, Zhang W, Gabler S, et al. Mapping the structure and function of the E1 and E2 glycoproteins in alphaviruses. Structure. 2006;14:63-73.

271. Munowitz MG, Dobson CM, Griffin RG, Harrison SC. On the rigidity of RNA in tomato bushy stunt virus. J Mol Biol. 1980;141:327-333.

272. Muramoto Y, Takada A, Fujii K, et al. Hierarchy among viral RNA (vRNA) segments in their role in vRNA incorporation into influenza A virions. J Virol. 2006;80:2318-2325.

273. Nakatsu S, Murakami S, Shindo K, et al. Influenza C and D viruses package eight organized ribonucleoprotein complexes. J Virol. 2018;92:e02084-17.

274. Nakatsu S, Sagara H, Sakai-Tagawa Y, Sugaya N, Noda T, Kawaoka Y. Complete and incomplete genome packaging of influenza A and B viruses. MBio. 2016;7.

275. Narayanan K, Maeda A, Maeda J, Makino S. Characterization of the coronavirus M protein and nucleocapsid interaction in infected cells. J Virol. 2000;74:8127-8134.

276. Nelson CD, Minkkinen E, Bergkvist M, et al. Detecting small changes and additional peptides in the canine parvovirus capsid structure. J Virol. 2008;82:10397-10407.

277. Neu U, Woellner K, Gauglitz G, Stehle T. Structural basis of GM1 ganglioside recognition by simian virus 40. Proc Natl Acad Sci U S A. 2008;105:5219-5224.

278. Newcomb WW, Homa FL, Thomsen DR, et al. Assembly of the herpes simplex virus capsid: characterization of intermediates observed during cell-free capsid formation. J Mol Biol. 1996;263:432-446.

279. Newcomb WW, Juhas RM, Thomsen DR, et al. The UL6 gene product forms the portal for entry of DNA into the herpes simplex virus capsid. J Virol. 2001;75:10923-10932.

280. Newton ND, Hardy JM, Modhiran N, et al. The structure of an infectious immature flavivirus redefines viral architecture and maturation. Sci Adv. 2021;7.

281. Nibert ML, Fields BN. A carboxy-terminal fragment of protein ml/m1C is present in infectious subvirion particles of mammalian reoviruses and is proposed to have a role in penetration. J Virol. 1992;66:6408-6418.

282. Nishimura H, Hara M, Sugawara K, et al. Characterization of the cord-like structures emerging from the surface of influenza C virus-infected cells. Virology. 1990;179:179-188.

283. Nishimura H, Hongo S, Sugawara K, et al. The ability of influenza C virus to generate cord-like structures is influenced by the gene coding for M protein. Virology. 1994;200:140-147.

284. Noda T, Sagara H, Yen A, et al. Architecture of ribonucleoprotein complexes in influenza A virus particles. Nature. 2006;439:490-492.

285. Noda T, Sugita Y, Aoyama K, et al. Three-dimensional analysis of ribonucleoprotein complexes in influenza A virus. Nat Commun. 2012;3:639.

286. Olia AS, Prevelige PE, Jr., Johnson JE, Cingolani G. Three-dimensional structure of a viral genome-delivery portal vertex. Nat Struct Mol Biol. 2011;18:597-603.

287. Ostapchuk P, Almond M, Hearing P. Characterization of Empty adenovirus particles assembled in the absence of a functional adenovirus IVa2 protein. J Virol. 2011;85:5524-5531.

288. Owen KE, Kuhn RJ. Alphavirus budding is dependent on the interaction between the nucleocapsid and hydrophobic amino acids on the cytoplasmic domain of the E2 envelope glycoprotein. Virology. 1997;230:187-196.

289. Ozorowski G, Pallesen J, de Val N, et al. Open and closed structures reveal allostery and pliability in the HIV-1 envelope spike. Nature. 2017;547:360-363.

290. Palese P, Tobita K, Ueda M, Compans RW. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. Virology. 1974;61:397-410.

291. Pan J, Peng H, Chen B, Harrison SC. Cryo-EM Structure of full-length HIV-1 Env bound with the Fab of antibody PG16. J Mol Biol. 2020;432:1158-1168.

292. Pan J, Qian X, Lattmann S, et al. Structure of the human metapneumovirus polymerase phosphoprotein complex. Nature. 2020;577:275-279.

293. Pan J, Vakharia VN, Tao YJ. The structure of a birnavirus polymerase reveals a distinct active site topology. Proc Natl Acad Sci U S A. 2007;104:7385-7390.

294. Pan M, Alvarez-Cabrera AL, Kang JS, Wang L, Fan C, Zhou ZH. Asymmetric reconstruction of mammalian reovirus reveals interactions among RNA, transcriptional factor micro2 and capsid proteins. Nat Commun. 2021;12:4176.

295. Pancera M, Changela A, Kwong PD. How HIV-1 entry mechanism and broadly neutralizing antibodies guide structure-based vaccine design. Curr Opin HIV AIDS. 2017;12:229-240.

296. Pancera M, Majeed S, Ban YE, et al. Structure of HIV-1 gp120 with gp41-interactive region reveals layered envelope architecture and basis of conformational mobility. Proc Natl Acad Sci U S A. 2010;107:1166-1171.

297. Panjwani A, Strauss M, Gold S, et al. Capsid protein VP4 of human rhinovirus induces membrane permeability by the formation of a size-selective multimeric pore. PLoS Pathog. 2014;10:e1004294.

298. Parent KN, Khayat R, Tu LH, et al. P22 coat protein structures reveal a novel mechanism for capsid maturation: stability without auxiliary proteins or chemical crosslinks. Structure. 2010;18:390-401.

299. Parker JS, Murphy WJ, Wang D, O'Brien SJ, Parrish CR. Canine and feline parvoviruses can use human or feline transferrin receptors to bind, enter, and infect cells. J Virol. 2001;75:3896-3902.

300. Parker JS, Parrish CR. Cellular uptake and infection by canine parvovirus involves rapid dynamin-regulated clathrin-mediated endocytosis, followed by slower intracellular trafficking. J Virol. 2000;74:1919-1930.

301. Paulson JC, Sadler JE, Hill RL. Restoration of specific myxovirus receptors to asialoerythrocytes by incorporation of sialic acid with pure sialyltransferases. J Biol Chem. 1979;254:2120-2124.

302. Peng R, Zhang S, Cui Y, Shi Y, Gao GF, Qi J. Structures of human-infecting Thogotovirus fusogens support a common ancestor with insect baculovirus. Proc Natl Acad Sci U S A. 2017;114:E8905-E8912.

303. Perez-Vargas J, Krey T, Valansi C, et al. Structural basis of eukaryotic cell-cell fusion. Cell. 2014;157:407-419.

304. Persson BD, Muller S, Reiter DM, et al. An arginine switch in the species B adenovirus knob determines high-affinity engagement of cellular receptor CD46. J Virol. 2009;83:673-686.

305. Peukes J, Xiong X, Briggs JAG. New structural insights into the multifunctional influenza A matrix protein 1. FEBS Lett. 2021;595:2535-2543.

306. Peukes J, Xiong X, Erlendsson S, et al. The native structure of the assembled matrix protein 1 of influenza A virus. Nature. 2020;587:495-498.

307. Pevear DC, Fancher MJ, Felock PJ, et al. Conformational change in the floor of the human rhinovirus canyon blocks adsorption to HeLa cell receptors. J Virol. 1989;63:2002-2007.

308. Piai A, Fu Q, Cai Y, et al. Structural basis of transmembrane coupling of the HIV-1 envelope glycoprotein. Nat Commun. 2020;11:2317.

309. Piai A, Fu Q, Sharp AK, Bighi B, Brown AM, Chou JJ. NMR model of the entire membrane-interacting region of the HIV-1 fusion protein and its perturbation of membrane morphology. J Am Chem Soc. 2021;143:6609-6615.

310. Pletnev SV, Zhang W, Mukhopadhyay S, et al. Locations of carbohydrate sites on alphavirus glycoproteins show that E1 forms an icosahedral scaffold. Cell. 2001;105:127-136.

311. Poranen MM, Tuma R, Bamford DH. Assembly of double-strand RNA viruses. In: Roy P, ed. Virus Structure and Assembly. Vol. 64. Elsevier Academic Press; 2005:15-43.

312. Pornillos O, Garrus JE, Sundquist WI. Mechanisms of enveloped RNA virus budding. Trends Cell Biol. 2002;12:569-579.

313. Prasad BV, Hardy ME, Dokland T, Bell J, Rossmann MG, Estes MK. X-ray crystallographic structure of the Norwalk virus capsid. Science. 1999;286:287-290.

314. Prasad VM, Miller AS, Klose T, et al. Structure of the immature Zika virus at 9 A resolution. Nat Struct Mol Biol. 2017;24:184-186.

315. Prevelige PE Jr, Cortines JR. Phage assembly and the special role of the portal protein. Curr Opin Virol. 2018;31:66-73.

316. Pulkkinen LIA, Barrass SV, Domanska A, Overby AK, Anastasina M, Butcher SJ. Molecular organisation of tick-borne encephalitis virus. Viruses. 2022;14.

317. Rand RP, Luzzati V. X-ray diffraction study in water of lipids extracted from human erythrocytes: the position of cholesterol in the lipid lamellae. Biophys J. 1968;8:125-137.

318. Raunser S. Cryo-EM Revolutionizes the Structure Determination of Biomolecules. Angew Chem Int Ed Engl. 2017;56:16450-16452.

319. Razinkov VI, Melikyan GB, Cohen FS. Hemifusion between cells expressing hemagglutinin of influenza virus and planar membranes can precede the formation of fusion pores that subsequently fully enlarge. Biophys J. 1999;77:3144-3151.

320. Reddy HK, Carroni M, Hajdu J, Svenda M. Electron cryo-microscopy of bacteriophage PR772 reveals the elusive vertex complex and the capsid architecture. Elife. 2019;8.

321. Reddy VS, Natarajan P, Okerberg B, et al. Virus Particle Explorer (VIPER), a website for virus capsid structures and their computational analyses. J Virol. 2001;75:11943-11947.

322. Reddy VS, Natchiar SK, Stewart PL, Nemerow GR. Crystal structure of human adenovirus at 3.5 A resolution. Science. 2010;329:1071-1075.

323. Reddy VS, Nemerow GR. Structures and organization of adenovirus cement proteins provide insights into the role of capsid maturation in virus entry and infection. Proc Natl Acad Sci U S A. 2014;111:11715-11720.

324. Reinisch K, Nibert M, Harrison SC. The reovirus core: structure of a complex molecular machine. Nature. 2000;404:960-967.

325. Reiter DM, Frierson JM, Halvorson EE, Kobayashi T, Dermody TS, Stehle T. Crystal structure of reovirus attachment protein sigma1 in complex with sialylated oligosaccharides. PLoS Pathog. 2011;7:e1002166.

326. Rey FA, Heinz FX, Mandl C, Kunz C, Harrison SC. The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. Nature. 1995;375:291-298.

327. Rixon FJ, Schmid MF. Structural similarities in DNA packaging and delivery apparatuses in Herpesvirus and dsDNA bacteriophages. Curr Opin Virol. 2014;5:105-110.

328. Roberts MM, White JL, Grütter MG, Burnett RM. Three-dimensional structure of the adenovirus major coat protein hexon. Science. 1986;232:1148-1151.

329. Robinson IK, Harrison SC. Structure of the expanded state of tomato bushy stunt virus. Nature (London). 1982;297:563-568.

330. Roche S, Bressanelli S, Rey FA, Gaudin Y. Crystal structure of the low-pH form of the vesicular stomatitis virus glycoprotein G. Science. 2006;313:187-191.

331. Roche S, Gaudin Y. Characterization of the equilibrium between the native and fusion-inactive conformation of rabies virus glycoprotein indicates that the fusion complex is made of several trimers. Virology. 2002;297:128-135.

332. Roche S, Rey FA, Gaudin Y, Bressanelli S. Structure of the prefusion form of the vesicular stomatitis virus glycoprotein G. Science. 2007;315:843-848.

333. Rolfsson O, Middleton S, Manfield IW, et al. Direct evidence for packaging signal-mediated assembly of bacteriophage MS2. J Mol Biol. 2016;428:431-448.

334. Rosenthal PB, Zhang X, Formanowski F, et al. Structure of the haemagglutinin-esterase-fusion glycoprotein of influenza C virus. Nature. 1998;396:92-96.

335. Rossman JS, Jing X, Leser GP, Lamb RA. Influenza virus M2 protein mediates ESCRT-independent membrane scission. Cell. 2010;142:902-913.

336. Rossmann MG. The canyon hypothesis. Hiding the host cell receptor attachment site on a viral surface from immune surveillance. J Biol Chem. 1989;264:14587-14590.

337. Rossmann MG, Arnold E, Erickson JW, et al. Structure of a human common cold virus and functional relationship to other picornaviruses. Nature. 1985;317:145-153.

338. Rossmann MG, He Y, Kuhn RJ. Picornavirus-receptor interactions. Trends Microbiol. 2002;10:324-331.

339. Rossmann MG, Mesyanzhinov VV, Arisaka F, Leiman PG. The bacteriophage T4 DNA injection machine. Curr Opin Struct Biol. 2004;14:171-180.

340. Rudolph MG, Kraus I, Dickmanns A, Eickmann M, Garten W, Ficner R. Crystal structure of the borna disease virus nucleoprotein. Structure. 2003;11:1219-1226.

341. Russell RJ, Haire LF, Stevens DJ, et al. The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. Nature. 2006;443:45-49.

342. Rux JJ, Kuser PR, Burnett RM. Structural and phylogenetic analysis of adenovirus hexons by use of high-resolution x-ray crystallographic, molecular modeling, and sequence-based methods. J Virol. 2003;77:9553-9566.

343. Salgado EN, Garcia Rodriguez B, Narayanaswamy N, Krishnan Y, Harrison SC. Visualization of calcium ion loss from rotavirus during cell entry. J Virol. 2018;92:e01327-18.

344. Salgado EN, Upadhyayula S, Harrison SC. Single-particle detection of transcription following rotavirus entry. J Virol. 2017;91:e00651-17.

345. San Martin C. Latest insights on adenovirus structure and assembly. Viruses. 2012;4:847-877.

346. Scheiffele P, Rietveld A, Wilk T, Simons K. Influenza viruses select ordered lipid domains during budding from the plasma membrane. J Biol Chem. 1999;274:2038-2044.

347. Schmidt F, Weisblum Y, Muecksch F, et al. Measuring SARS-CoV-2 neutralizing antibody activity using pseudotyped and chimeric viruses. J Exp Med. 2020;217:e20201181.

348. Schur FK, Obr M, Hagen WJ, et al. An atomic model of HIV-1 capsid-SP1 reveals structures regulating assembly and maturation. Science. 2016;353:506-508.

349. Seiradake E, Henaff D, Wodrich H, et al. The cell adhesion molecule “CAR” and sialic acid on human erythrocytes influence adenovirus in vivo biodistribution. PLoS Pathog. 2009;5:e1000277.

350. Settembre EC, Chen JZ, Dormitzer PR, Grigorieff N, Harrison SC. Atomic model of an infectious rotavirus particle. EMBO J. 2010;30:408-416.

351. Sevvana M, Long F, Miller AS, et al. Refinement and analysis of the mature Zika virus Cryo-EM structure at 3.1 A resolution. Structure. 2018;26:1169-1177 e3.

352. Shaik MM, Peng H, Lu J, et al. Structural basis of coreceptor recognition by HIV-1 envelope spike. Nature. 2019;565:318-323.

353. Shakeel S, Dykeman EC, White SJ, et al. Genomic RNA folding mediates assembly of human parechovirus. Nat Commun. 2017;8:5.

354. Shaw JG, Plaskitt KA, Wilson TMA. Evidence that tobacco mosaic virus particles disassemble cotranslationally in vivo. Virology. 1986;148:326-336.

354a. Shi W, Cai Y, Zhu H., et al. Cryo-EM structure of SARS-CoV-2 postfusion spike in membrane. bioRxiv/10.1101/2022.12.05.519151v1.

355. Shields SA, Brisco MJ, Wilson TM, Hull R. Southern bean mosaic virus RNA remains associated with swollen virions during translation in wheat germ cell-free extracts. Virology. 1989;171:602-606.

356. Siegel DP. Energetics of intermediates in membrane fusion: comparison of stalk and inverted micellar intermediate mechanisms. Biophys J. 1993;65:2124-2140.

357. Silvestry M, Lindert S, Smith JG, et al. Cryo-electron microscopy structure of adenovirus type 2 temperature-sensitive mutant 1 reveals insight into the cell entry defect. J Virol. 2009;83:7375-7383.

358. Simpson AA, Tao Y, Leiman PG, et al. Structure of the bacteriophage phi29 DNA packaging motor. Nature. 2000;408:745-750.

359. Sirohi D, Chen Z, Sun L, et al. The 3.8 A resolution cryo-EM structure of Zika virus. Science. 2016;352:467-470.

360. Skehel JJ, Bayley PM, Brown EB, et al. Changes in the conformation of influenza virus haemagglutinin at the pH optimum of virus-mediated membrane fusion. Proc Natl Acad Sci U S A. 1982;79:968-972.

361. Skehel JJ, Wharton S, Calder LJ, Stevens D. On the activation of membrane fusion by influenza haemagglutinin. Novartis Foundation Symposium. John Wiley; 2008:56-64.

362. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem. 2000;69:531-569.

363. Smith DE, Tans SJ, Smith SB, Grimes S, Anderson DL, Bustamante C. The bacteriophage straight phi29 portal motor can package DNA against a large internal force. Nature. 2001;413:748-752.

364. Smith TJ, Kremer MJ, Luo M, et al. The site of attachment in human rhinovirus 14 for antiviral agents that inhibit uncoating. Science. 1986;233:1286-1293.

365. Stehle T, Gamblin SJ, Yan Y, Harrison SC. The structure of simian virus 40 refined at 3.1A resolution. Structure. 1996;4:165-182.

366. Stehle T, Yan Y, Benjamin TL, Harrison SC. Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. Nature. 1994;369:160-163.

367. Stewart PL, Burnett RM, Cyrklaff M, Fuller SD. Image reconstruction reveals the complex molecular organization of adenovirus. Cell. 1991;67:145-154.

368. Stewart PL, Fuller SD, Burnett RM. Difference imaging of adenovirus: bridging the resolution gap between X-ray crystallography and electron microscopy. EMBO J. 1993;12:2589-2599.

369. Stockley PG, Rolfsson O, Thompson GS, et al. A simple, RNA-mediated allosteric switch controls the pathway to formation of a T=3 viral capsid. J Mol Biol. 2007;369:541-552.

370. Strack B, Calistri A, Accola MA, Palu G, Gottlinger HG. A role for ubiquitin ligase recruitment in retrovirus release. Proc Natl Acad Sci U S A. 2000;97:13063-13068.

371. Strandin T, Hepojoki J, Vaheri A. Cytoplasmic tails of bunyavirus Gn glycoproteins-Could they act as matrix protein surrogates? Virology. 2013;437:73-80.

372. Sun S, Kondabagil K, Draper B, et al. The structure of the phage T4 DNA packaging motor suggests a mechanism dependent on electrostatic forces. Cell. 2008;135:1251-1262.

373. Sun S, Kondabagil K, Gentz PM, Rossmann MG, Rao VB. The structure of the ATPase that powers DNA packaging into bacteriophage T4 procapsids. Mol Cell. 2007;25:943-949.

374. Sun S, Rao VB, Rossmann MG. Genome packaging in viruses. Curr Opin Struct Biol. 2010;20:114-120.

375. Sundquist WI, Krausslich HG. HIV-1 assembly, budding, and maturation. Cold Spring Harb Perspect Med. 2012;2:a006924.

376. Supekar VM, Bruckmann C, Ingallinella P, Bianchi E, Pessi A, Carfi A. Structure of a proteolytically resistant core from the severe acute respiratory syndrome coronavirus S2 fusion protein. Proc Natl Acad Sci U S A. 2004;101:17958-17963.

377. Takeda M, Leser GP, Russell CJ, Lamb RA. Influenza virus hemagglutinin concentrates in lipid raft microdomains for efficient viral fusion. Proc Natl Acad Sci U S A. 2003;100:14610-14617.

378. Tang J, Lander GC, Olia AS, et al. Peering down the barrel of a bacteriophage portal: the genome packaging and release valve in p22. Structure. 2011;19:496-502.

379. Tao Y, Farsetta DL, Nibert ML, Harrison SC. RNA synthesis in a cage—structural studies of reovirus polymerase lambda3. Cell. 2002;111:733-745.

380. Tao Y, Olson NH, Xu W, Anderson DL, Rossmann MG, Baker TS. Assembly of a tailed bacterial virus and its genome release studied in three dimensions. Cell. 1998;95:431-437.

381. Taraporewala ZF, Jiang X, Vasquez-Del Carpio R, Jayaram H, Prasad BV, Patton JT. Structure-function analysis of rotavirus NSP2 octamer by using a novel complementation system. J Virol. 2006;80:7984-7994.

382. Taylor GM, Hanson PI, Kielian M. Ubiquitin depletion and dominant-negative VPS4 inhibit rhabdovirus budding without affecting alphavirus budding. J Virol. 2007;81:13631-13639.

383. Taylor NMI, van Raaij MJ, Leiman PG. Contractile injection systems of bacteriophages and related systems. Mol Microbiol. 2018;108:6-15.

384. Therkelsen MD, Klose T, Vago F, Jiang W, Rossmann MG, Kuhn RJ. Flaviviruses have imperfect icosahedral symmetry. Proc Natl Acad Sci U S A. 2018;115:11608-11612.

385. Timmins J, Ruigrok RW, Weissenhorn W. Structural studies on the Ebola virus matrix protein VP40 indicate that matrix proteins of enveloped RNA viruses are analogues but not homologues. FEMS Microbiol Lett. 2004;233:179-186.

386. Tsang SK, McDermott BM, Racaniello VR, Hogle JM. Kinetic analysis of the effect of poliovirus receptor on viral uncoating: the receptor as a catalyst. J Virol. 2001;75:4984-4989.

387. Turner DR, Joyce LE, Butler PJG. The tobacco mosaic virus assembly origin RNA. J Mol Biol. 1988;203:531-547.

388. Tuthill TJ, Bubeck D, Rowlands DJ, Hogle JM. Characterization of early steps in the poliovirus infection process: receptor-decorated liposomes induce conversion of the virus to membrane-anchored entry-intermediate particles. J Virol. 2006;80:172-180.

389. Valegard K, Liljas L, Fridborg K, Unge T. The three-dimensional structure of the bacterial virus MS2. Nature. 1990;345:36-41.

390. Valegard K, Murray JB, Stockley PG, Stonehouse NJ, Liljas L. Crystal structure of an RNA bacteriophage coat protein-operator complex. Nature. 1994;371:623-626.

391. Valegard K, Murray JB, Stonehouse NJ, van den Worm S, Stockley PG, Liljas L. The three-dimensional structures of two complexes between recombinant MS2 capsids and RNA operator fragments reveal sequence-specific protein-RNA interactions. J Mol Biol. 1997;270:724-738.

392. van den Pol AN, Mao G, Chattopadhyay A, Rose JK, Davis JN. Chikungunya, influenza, Nipah, and Semliki forest chimeric viruses with vesicular stomatitis virus: actions in the brain. J Virol. 2017;91:e02154-16.

393. Vogel RH, Provencher SW, von Bonsdorff CH, Adrian M, Dubochet J. Envelope structure of Semliki Forest virus reconstructed from cryo-electron micrographs. Nature. 1986;320:533-535.

394. Vollmer B, Prazak V, Vasishtan D, et al. The prefusion structure of herpes simplex virus glycoprotein B. Sci Adv. 2020;6:eabc1726.

395. Von Bonsdorff CH, Harrison SC. Sindbis virus glycoproteins form a regular icosahedral surface lattice. J Virol. 1975;16:141-145.

396. Von Bonsdorff CH, Pettersson RF. Surface structure of Uukuniemi virus. J Virol. 1975;16:1296-1307.

397. von Itzstein M, Wu W-Y, Kok GB, et al. Rational design of potent sialidase-based inhibitors of influenza virus replication. Nature. 1993;363:418-423.

398. Votteler J, Sundquist WI. Virus budding and the ESCRT pathway. Cell Host Microbe. 2013;14:232-241.

399. Wan W, Briggs JA. Cryo-electron tomography and subtomogram averaging. Methods Enzymol. 2016;579:329-367.

400. Ward AB, Wilson IA. The HIV-1 envelope glycoprotein structure: nailing down a moving target. Immunol Rev. 2017;275:21-32.

401. Wei H, Cheng RH, Berriman J, et al. Three-dimensional structure of the enveloped bacteriophage phi12: an incomplete T = 13 lattice is superposed on an enclosed T = 1 shell. PLoS One. 2009;4:e6850.

402. Weis W, Brown J, Cusack S, Paulson JE, Skehel JJ, Wiley DC. The structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. Nature (London). 1988;333:426-431.

403. Weissenhorn W, Carfi A, Lee KH, Skehel JJ, Wiley DC. Crystal structure of the Ebola virus membrane fusion subunit, GP2, from the envelope glycoprotein ectodomain. Mol Cell. 1998;2:605-616.

404. Weissenhorn W, Dessen A, Harrison SC, Skehel JJ, Wiley DC. Atomic structure of the ectodomain from HIV-1 gp41. Nature. 1997;387:426-430.

405. Weissenhorn W, Poudevigne E, Effantin G, Bassereau P. How to get out: ssRNA enveloped viruses and membrane fission. Curr Opin Virol. 2013;3:159-167.

406. Welker L, Paillart JC, Bernacchi S. Importance of viral late domains in budding and release of enveloped RNA viruses. Viruses. 2021;13:1559.

407. Whittle JR, Zhang R, Khurana S, et al. Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. Proc Natl Acad Sci U S A. 2011;108:14216-14221.

408. Wiethoff CM, Wodrich H, Gerace L, Nemerow GR. Adenovirus protein VI mediates membrane disruption following capsid disassembly. J Virol. 2005;79:1992-2000.

409. Wikoff WR, Liljas L, Duda RL, Tsuruta H, Hendrix RW, Johnson JE. Topologically linked protein rings in the bacteriophage HK97 capsid. Science. 2000;289:2129-2133.

410. Wiley DC, Skehel JJ. Crystallization and x-ray diffraction studies on the haemagglutinin glycoprotein from the membrane of influenza virus. J Mol Biol. 1977;112: 343-347.

411. Willensky S, Bar-Rogovsky H, Bignon EA, Tischler ND, Modis Y, Dessau M. Crystal structure of glycoprotein C from a Hantavirus in the post-fusion conformation. PLoS Pathog. 2016;12:e1005948.

412. Wilson IA, Skehel JJ, Wiley DC. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. Nature (London). 1981;289:366-373.

413. Wilson TMA. Cotranslational disassembly of tobacco mosaic virus in vitro. Virology. 1984;137:255-265.

414. Wirblich C, Bhattacharya B, Roy P. Nonstructural protein 3 of bluetongue virus assists virus release by recruiting ESCRT-I protein Tsg101. J Virol. 2006;80:460-473.

415. Wolf M, Garcea RL, Grigorieff N, Harrison SC. Subunit interactions in bovine papillomavirus. Proc Natl Acad Sci U S A. 2010;107:6298-6303.

416. Wrapp D, Wang N, Corbett KS, et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. Science. 2020;367:1260-1263.

417. Xiao T, Frey G, Fu Q, et al. HIV-1 fusion inhibitors targeting the membrane-proximal external region of Env spikes. Nat Chem Biol. 2020;16:529-537.

418. Yang Z, Wang H, Liu AZ, Gristick HB, Bjorkman PJ. Asymmetric opening of HIV-1 Env bound to CD4 and a coreceptor-mimicking antibody. Nat Struct Mol Biol. 2019;26:1167-1175.

419. Ye Q, Krug RM, Tao YJ. The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA. Nature. 2006;444:1078-1082.

420. Yin HS, Paterson RG, Wen X, Lamb RA, Jardetzky TS. Structure of the uncleaved ectodomain of the paramyxovirus (hPIV3) fusion protein. Proc Natl Acad Sci U S A. 2005;102:9288-9293.

421. Yin HS, Wen X, Paterson RG, Lamb RA, Jardetzky TS. Structure of the parainfluenza virus 5 F protein in its metastable, prefusion conformation. Nature. 2006;439:38-44.

422. Yoder JD, Dormitzer PR. Alternative intermolecular contacts underlie the rotavirus VP5(*) two- to three-fold rearrangement. Embo J. 2006;25(7):1559-1568.

423. Yu IM, Holdaway HA, Chipman PR, Kuhn RJ, Rossmann MG, Chen J. Association of the pr peptides with dengue virus at acidic pH blocks membrane fusion. J Virol. 2009;83:12101-12107.

424. Yu IM, Zhang W, Holdaway HA, et al. Structure of the immature dengue virus at low pH primes proteolytic maturation. Science. 2008;319:1834-1837.

425. Yu X, Mullen TM, Abrishami V, Huiskonen JT, Nemerow GR, Reddy VS. Structure of a cell entry defective human adenovirus provides insights into precursor proteins and capsid maturation. J Mol Biol. 2022;434:167350.

426. Yuan S, Wang J, Zhu D, et al. Cryo-EM structure of a herpesvirus capsid at 3.1 A. Science. 2018;360.

427. Yuan Y, Cao D, Zhang Y, et al. Cryo-EM structures of MERS-CoV and SARS-CoV spike glycoproteins reveal the dynamic receptor binding domains. Nat Commun. 2017;8:15092.

428. Zamora PF, Hu L, Knowlton JJ, et al. Reovirus nonstructural protein sigmaNS acts as an RNA stability factor promoting viral genome replication. J Virol. 2018;92:e00563-18.

429. Zhang J, Pekosz A, Lamb RA. Influenza virus assembly and lipid raft microdomains: a role for the cytoplasmic tails of the spike glycoproteins. J Virol. 2000;74:4634-4644.

430. Zhang J, Xiao T, Cai Y, Chen B. Structure of SARS-CoV-2 spike protein. Curr Opin Virol. 2021;50:173-182.

431. Zhang L, Agosto MA, Ivanovic T, King DS, Nibert ML, Harrison SC. Requirements for the formation of membrane pores by the reovirus myristoylated micro1N peptide. J Virol. 2009;83:7004-7014.

432. Zhang R, Hryc CF, Cong Y, et al. 4.4 A cryo-EM structure of an enveloped alphavirus Venezuelan equine encephalitis virus. EMBO J. 2011;30:3854-3863.

433. Zhang W, Imperiale MJ. Requirement of the adenovirus IVa2 protein for virus assembly. J Virol. 2003;77:3586-3594.

434. Zhang W, Low JA, Christensen JB, Imperiale MJ. Role for the adenovirus IVa2 protein in packaging of viral DNA. J Virol. 2001;75:10446-10454.

435. Zhang W, Mukhopadhyay S, Pletnev SV, Baker TS, Kuhn RJ, Rossmann MG. Placement of the structural proteins in Sindbis virus. J Virol. 2002;76:11645-11658.

436. Zhang X, Ding K, Yu X, Chang W, Sun J, Zhou ZH. In situ structures of the segmented genome and RNA polymerase complex inside a dsRNA virus. Nature. 2015;527:531-534.

437. Zhang X, Ge P, Yu X, et al. Cryo-EM structure of the mature dengue virus at 3.5-A resolution. Nat Struct Mol Biol. 2013;20:105-110.

438. Zhang X, Ji Y, Zhang L, et al. Features of reovirus outer capsid protein mu1 revealed by electron cryomicroscopy and image reconstruction of the virion at 7.0 Angstrom resolution. Structure. 2005;13:1545-1557.

439. Zhang Y, Corver J, Chipman PR, et al. Structures of immature flavivirus particles. EMBO J. 2003;22:2604-2613.

440. Zhao H, Lindqvist B, Garoff H, von Bonsdorff CH, Liljestrom P. A tyrosine-based motif in the cytoplasmic domain of the alphavirus envelope protein is essential for budding. EMBO J. 1994;13:4204-4211.

441. Zheng Q, Deng Y, Liu J, van der Hoek L, Berkhout B, Lu M. Core structure of S2 from the human coronavirus NL63 spike glycoprotein. Biochemistry. 2006;45:15205-15215.

442. Zhou K, Si Z, Ge P, Tsao J, Luo M, Zhou H. Atomic model of vesciular stomatitis virus and mechanism of assembly. Nat Commun. 2022;13(1):5980. bioRxiv 2022.05.02.490251.

443. Zhu ZH, Chen SS, Huang AS. Phenotypic mixing between human immunodeficiency virus and vesicular stomatitis virus or herpes simplex virus. J Acquir Immune Defic Syndr (1988). 1990;3:215-219.

444. Zimmern D, Wilson TMA. Location of the origin for viral reassembly on tobacco mosaic virus RNA and its relation to stable fragment. FEBS Lett. 1976;71:294-298.

445. Zubieta C, Schoehn G, Chroboczek J, Cusack S. The structure of the human adenovirus 2 penton. Mol Cell. 2005;17:121-135.

CHAPTER 4

Virus Entry and Uncoating

Jason Mercer

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Introduction

Viral particles have a single mission: to transport the viral genome from an infected host cell to a noninfected host cell and to deliver it into the cytoplasm or the nucleus in a replication-competent form. The target can be a neighboring cell, a cell elsewhere in the host organism, or a cell in another organism. The process starts in an infected cell with the packaging of the viral genome and accessory proteins into a new virus particle, which is released into the extracellular space. When the virus contacts the surface of a new host cell, a complex series of events ensues tightly coordinated in time and space. These events include binding to receptors and signaling, often followed by endocytic internalization, vesicular trafficking, membrane penetration, cytosolic transport, and nuclear import (Fig. 4.1). Uncoating is an integral part of the process; the virus particle is modified, destabilized, disassembled, and eventually the genome, present in a protected and condensed form in the virion, is decondensed and exposed in a replication- or transcription-competent form. The progression of a virus particle through its entry program depends critically on cellular functions. The *Trojan horse strategy* that is used is necessary because the particles are simple and capable of limited independent functions.

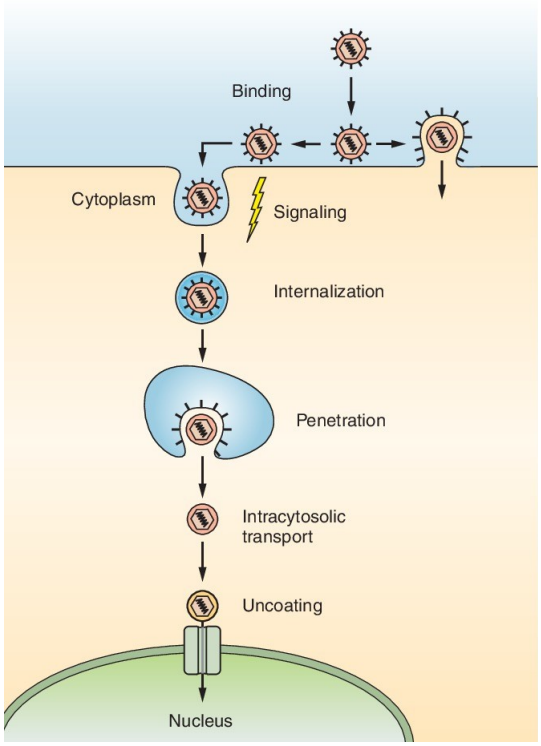

FIGURE 4.1 **Stepwise entry of animal viruses**. The entry of animal viruses involves a series of steps that start with virus binding to the cell surface. Binding is multivalent and involves cell surface molecules serving as passive attachment factors or receptors that are more active in that they activate signaling pathways, induce conformational changes in the virus, or mediate endocytic internalization. Although some enveloped viruses are able to fuse with the plasma membrane, the majority of viruses rely on internalization by different endocytic mechanisms. Internalization is followed by transport to secondary organelles (usually endosomes), where the virus receives cues to trigger the penetration process. After reaching the cytosol, the viruses or viral capsids are transported to the site of genome uncoating and replication. For most DNA viruses and a few RNA viruses, this site is the nucleus. Many viruses have evolved mechanisms that allow them to deliver their genome and accessory proteins through the nuclear pore complexes.

This chapter describes some of the general concepts that govern cellular entry of animal viruses. For information about the entry of specific viruses and virus families, the reader is referred to the virus chapters. Information relevant to the topics covered here also can be found in numerous reviews that cover early virus cell interactions.[59,114,149,159,160,177,211,231,291]

The Barriers

The first barrier that incoming viruses must overcome is the glycocalyx, a layer of glycoconjugates that covers the external surface of cells. It is composed of glycoproteins, glycolipids, and proteoglycans. The composition and thickness of this layer is variable. By binding to oligosaccharides, many viruses make use of the glycocalyx for initial attachment.

The next barrier is the plasma membrane. Responsible for the cell's exchanges with the environment, it is the most complex and most dynamic of all cell membranes. The composition and properties are regulated by the endocytic and secretory pathways and by a continuous association and disassociation of proteins that interact with the cytosolic leaflet. The plasma membrane is a highly sensitive organ for recognizing and responding to external stimuli. Viruses take advantage of this during entry.

After clearing the plasma membrane by direct penetration or by exploiting endocytic pathways, viruses and viral capsids have to reach sites deeper in the cytoplasm. The cortical actin network underneath the plasma membrane and extreme crowding constitute major barriers to movement within the cytoplasm.[253] Finally, because many viruses replicate in the nucleus, the genome and accessory proteins must travel to the nucleus and cross the nuclear envelope. This requires cooperation between the incoming virus and the nuclear import machinery.[290]

Virus Binding to the Cell Surface

Viruses can only infect cells to which they can bind. Binding occurs to *attachment factors* and *virus receptors* on the surface of the cell. To a large extent, the identity, distribution, and behavior of these cellular components determine which cell types, tissues, and organisms a virus can infect. The receptors also define, in part, the pathogenic potential of a virus as well the nature of the disease that it causes.

Virus *receptors* can be defined as cell surface molecules that bind the incoming viruses to the cell and, in addition, promote entry by (1) inducing conformational changes in the virus that lead to priming, association with other receptors, membrane fusion, and penetration; (2) transmitting signals through the plasma membrane that lead to virus uptake or penetration and prepare the cell for the invasion; or (3) guiding bound virus particles into a variety of endocytic pathways.[177] *Attachment factors* help to concentrate the particles on the cell's surface, thus enhancing entry and infection. Unlike receptors, however, they do not actively promote entry and mediate signals. Often, the interactions with attachment factors are not highly specific. In practice, the distinction between a receptor and an attachment factor is not always straightforward because the consequences of virus binding to a given surface component are difficult to assess experimentally and can vary depending on cell type and conditions.

Receptors and attachment factors constitute a diverse collection of proteins, carbohydrates, and lipids with physiologic functions unrelated to pathogen interaction. Ranging from abundant and ubiquitous to rare and species specific, they differ from one virus to the next. In the past few decades, an impressive number have been identified in different virus and host cell systems. As shown in Table 4.1, which gives a partial list, receptors include ligand-binding receptors, glycoproteins, ion channels, gangliosides, carbohydrates, proteoglycans, and so on. Some families of surface molecules seem to be favored; the immunoglobulin-G superfamily of transmembrane proteins, proteoglycans, and glycoconjugates with terminal sialic acid residues, belong to these. In addition, a large group of viruses use integrins as their receptors.[260]

TABLE 4.1 Receptor proteins for some viruses

Virus	Family	Receptor	Function	References
G-protein–coupled receptors				
HIV	*Retroviridae*	CXCR4,CCR3,CCR2b,CCR8 CCR5	Chemokine receptors	2,46,68,227
HIV/SIV	*Retroviridae*	CCR5, Bonzo/STRL-33/ TYMSTR, BOB/GPR15, GPR1	Chemokine receptors	3,80,148
Proteins with multiple membrane-spanning domains				
GALV/FeLV-B/SSAV	*Retroviridae*	PiT-1	Phosphate transport	194,270
MLV-E	*Retroviridae*	MCAT-1	Cationic amino acid transport	1
MLV-A	*Retroviridae*	PiT-2	Phosphate transport	179,280
MLV-X/MLV-P	*Retroviridae*	XPR1/Rmc1/SYG1	Transporter	13,269
HCV	*Flaviviridae*	CD81	Tetraspanin membrane protein	205
Enterovirus 71	*Picornaviridae*	SCARB2	Endosomal membrane protein	299
Ebola virus	*Filoviridae*	NPC1	Endo/lysosomal cholesterol transporter	38,52
Immunoglobulin-related proteins				
Poliovirus	*Picornaviridae*	PVR (CD155)	Adhesion receptor	172
PRV/BHV-1	*Herpesviridae*	PVR (CD155)	Adhesion receptor	94
HSV-1/HSV-2/PRV	*Herpesviridae*	Prr2/HveB/nectin-2	Adhesion	79
HSV-/HSV-2/	*Herpesviridae*	Prr1/HveC/nectin-1	Adhesion	94
Coxsackie B	*Picornaviridae*	CAR	Homotypic cell interaction	17,272
Ad-2/Ad-5	*Adenoviridae*	CAR	Homotypic cell interaction	18,272
MHV-A59	*Coronaviridae*	MHVR/Bgp1 (a)	Biliary glycoprotein	72
Human rhinoviruses (type B and A major group)	*Picornaviridae*	ICAM-1	Cell adhesion/signaling	100,258
HIV/SIV	*Retroviridae*	CD4	T-cell signaling	156
HHV-7	*Herpesviridae*	CD4	T-cell signaling	153
AAV2	*Parvoviridae*	AAVR	Type I transmembrane protein	206
Low-density lipoprotein receptor–related proteins				
Rous sarcoma virus (type A)	*Retroviridae*	LDLR	Lipoprotein receptor	12
Human rhinoviruses (type A, minor group)	*Picornaviridae*	LDLR/α 2MR/LRP	Lipoprotein receptors	111
Venezuelan equine encephalitis virus	*Togaviridae*	LDLRAD3	Lipoprotein receptor	155
Integrins				
Adenovirus	*Adenoviridae*	$\alpha v\beta 3$	Vitronectin binding	292
Coxsackie A9	*Picornaviridae*	$\alpha v\beta 3$	Vitronectin binding	223
Adenovirus	*Adenoviridae*	$\alpha v\beta 5$	Vitronectin binding	293
Echoviruses-1/-8	*Picornaviridae*	$\alpha 2\beta 1$Banks	Collagen/laminin binding	20
Foot and mouth disease virus	*Picornaviridae*	$\alpha 2\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 6$	Vitronectin binding	22,117
Hantaan virus	*Bunyaviridae*	$\alpha 3$ integrins		90
Rotavirus	*Reoviridae*	$\alpha 4\beta 1$, $\alpha v\beta 3$, $\alpha 2\beta 1$		109
Cytomegalovirus	*Herpesviridae*	$\alpha v\beta 3$, $\alpha 2\beta 1$, $\alpha 6\beta 1$		82
Tumor necrosis factor receptor–related proteins				
ALV-B/D/E	*Retroviridae*	TVB	Apoptosis-inducing receptor	28
Herpes simplex virus 1	*Herpesviridae*	HveA	LIGHT receptor	39,165
Small consensus repeat–containing proteins				

Epstein-Barr virus	*Herpesviridae*	CR2	C3d/C3dg/iC3b binding	84,85
Measles	*Paramyxoviridae*	CD46	Complement inhibition	69
Echoviruses	*Picornaviridae*	CD55, neonatal Fc receptor	Complement inhibition/Fc receptor	17,182,306
Coxsackie B-1/-3/-5	*Picornaviridae*	CD55	Complement inhibition	19,241
Miscellaneous				
LCMV/Lassa fever virus	*Arenaviridae*	α-Dystroglycan	Laminin/agrin binding	36
Coronavirus-229E/TGEV	*Coronaviridae*	Aminopeptidase-*N*	Metalloproteinase	19,300
SARS-CoV/SARS-CoV-2	*Coronaviridae*	ACE2	Metallocarboxypeptidase	112,144
Arthritogenic alphaviruses	*Togaviridae*	Mxra8	Matrix/signaling modulator	303

Many viruses use multiple attachment factors and receptors. They interact with them in parallel or in series, or they use different receptors for entry into different cell types. When multiple receptors are required for productive entry, it is the convention to call the one contacted first a *receptor* and the subsequent ones *coreceptors*. A good example is human immunodeficiency virus type 1 (HIV-1), which makes use of heparan sulfate proteoglycans as attachment factors, CD4 as a receptor,[37] and CXCR4 and CCR5 (or a related chemokine receptor) as coreceptors.[21,181] In this case, the two receptors are used to induce stepwise conformational changes in the spike glycoprotein. For fusion, the hemorrhagic fever viruses, Ebola and Lassa, each engage two different receptors, one on the plasma membrane and one within endocytic carrier compartments. In both cases, binding to the primary receptor at the cell surface triggers virus uptake by endocytosis.[4,135] Within endosomal compartments, a low pH-dependent conformational change in the viral glycoproteins facilitate a receptor switch that enables endosomal escape.[38,119,143] In other cases (eg, adenovirus 2 and coxsackie B virus), two receptors seem to be needed to overcome anatomic and topological barriers.[54,186] Herpes viruses and poxviruses are able to infect a variety of cell types, probably in part because they possess proteins that can bind to several different receptors.[86,255] Cases are also seen where unrelated viruses make use of the same receptors. One well-studied example is coxsackie and adenovirus receptor (CAR) (Table 4.1), which is used as a receptor both by adenovirus 2 and 5 and coxsackie B viruses.[304]

The interaction between a viral surface protein and a receptor can be highly specific, but the affinity is often low. However, the presence of multiple, closely spaced binding sites on the surface of the virus particle allows multivalent binding, and the avidity is, therefore, frequently high. The affinity of influenza hemagglutinin for sialic acid containing glycoconjugates is, for example, in the millimolar range, but virus binding to cells is virtually irreversible.[249] That most receptor molecules are laterally mobile in the plasma membrane allows, moreover, the formation of a local *microdomain* rich in receptors under the bound virus with a composition and properties different from that in the surrounding membrane.[75] The consequences can be the inclusion of the virus in lipid rafts, or entrapment in caveolae, coated pits, and other membrane specializations. Receptor clustering can also lead to transmembrane signaling, changes in the actin cytoskeleton, and recruitment of cytosolic factors such as a clathrin coat to the plasma membrane.

Although the interaction between a virus and its receptors is generally direct, there are cases where adaptor proteins are involved. Binding of antibodies to dengue virus allows infection of macrophages via Fc receptors on the surface of these cells.[201] In this case, the virus particle is internalized as an immune complex. Instead of neutralizing the virus, the antibodies mediate expansion of the host cell repertoire. Some Flaviviruses (including dengue), Arenaviruses, Poxviruses, and Alphaviruses employ a strategy termed apoptotic mimicry, whereby bridging molecules link phosphatidylserine in the virus envelope with cellular phosphatidylserine receptors (reviewed in Ref.[5]). This strategy serves to trigger virus entry and simultaneously dampen host immune responses to infection.[24,168,174,242]

In enveloped viruses, the spike glycoproteins are responsible for receptor binding. Typically, these are oligomeric type 1 integral membrane proteins that have the bulk of their mass outside the membrane with the receptor-binding domain exposed. Other external domains in the same protein may be responsible for membrane fusion and receptor destruction. In some spike proteins (eg, the HIV-1 glycoprotein), the receptor-binding moiety is not covalently connected to the rest, which allows its dissociation once receptor interaction has occurred. X-ray crystal structures of spike glycoprotein-receptor complexes exist for several enveloped viruses (see Chapter 3).

In nonenveloped viruses, the structures that bind receptors are projections or indentations in the capsid surface. Adenoviruses have trimeric fiber proteins with globular knobs that project from the vertices.[23] The penton base protein of many adenovirus subfamilies contains in addition an exposed Arg-Gly-Asp (RGD) sequence that associates with integrins.[259] Many enterovirus receptors bind in a cleft in the capsid surface called the *canyon*, the molecular features of which have been analyzed in great detail.[225]

Role of Carbohydrates

Glycoconjugates on the cell surface have an important role during entry of many viruses as receptors and attachment factors. Glycoproteins and glycolipids, with terminal sialic-acid residues, serve as specific receptors for a variety of viruses, including orthomyxo-, paramyxo-, and polyoma viruses. The HA1 subunits of influenza A virus hemagglutinin (HA) bind terminal sialic acid residues associated with galactose through either a Neu5Ac α(2,3)-Gal or Neu5Ac α(2,6)-Gal bond.[249] Human influenza recognizes the α(2,6) linkage; avian and equine viruses, the α(2,3) linkages, whereas porcine viruses appear to recognize both. These specificities reflect the structure of the glycans expressed in the different species and play a central role in limiting cross-species transmission. The tetrameric hemagglutinin-neuraminidase (HN) proteins of parainfluenza virus 5 has specificity for α(2,3)-sialyllactose,[301] and polyomaviruses bind to specific saccharide residues in the glycan moieties of various gangliosides.[276] A difference limited to a single atom in sialic acids plays a major role in species specificity of simian virus 40 (SV40), because it binds better to the simian GM1 ganglioside, which has a *N*-glycolylneuraminic acid, than to the human, which has a *N*-acetylneuraminic acid.[35]

The list of viruses recognized as binding to glycosaminoglycan (GAG) chains (eg, heparan- and chondroitin-sulfate) is steadily growing.[11] It now includes several herpes-, alpha-, flavi-, retro-, parvo-, picorna-, pox-, and papillomaviruses. Binding often involves positively charged patches in viral surface proteins. In some cases, viruses adopt GAGs as receptors when grown in tissue culture; their surface proteins mutate and express more basic residues.[34,250] In contrast to tissue culture–adapted strains, natural isolates do not necessarily bind to GAGs. Indeed, adaptation of

different glycan receptors is likely part of the age-old war against pathogens, including viruses and their hosts, a war in which the diversity of surface carbohydrates plays an important role. Poxviruses, for example, encode one chondroitin sulfate and two heparan sulfate binding proteins, yet viral binding protein dependence and GAG usage vary with virus species and strain.[14,15]

In most cases, it is the viruses that recognize host cell glycans. However, the reverse is true when cell surface lectins bind to glycans present in the envelope proteins of incoming viruses. One such lectin is DC-SIGN, a tetrameric, C-type lectin present on the surface of immature dendritic cells. It binds *N*-linked glycans of the high-mannose type,[81] such as in glycans that have failed to undergo terminal glycosylation in the Golgi complex of the infected cells. Because glycoproteins synthesized in insect cells have exclusively high-mannose glycans, viruses introduced into the skin via insect bites are often recognized by DC-SIGN, resulting in the infection of dendritic cells. Viruses that bind to these lectins include HIV-1, Sindbis, human cytomegalovirus, dengue, and severe acute respiratory syndrome (SARS) viruses.[92,133,152,209,247,271] Thus, cells that our body uses in the front-line defense against pathogens end up serving the interests of viruses instead by spreading the infection.

Mobility of Cell-Associated Viruses

The encounter between individual viruses and the cell can be visualized live by light microscopy using fluorescent viruses. What happens depends on the virus, the receptor, and the host cell. Parvovirus particles undergo rapid binding and release events that eventually result in permanent attachment and endocytic internalization.[239] Polyomavirus particles bind firmly and diffuse laterally in the membrane for 5-10 seconds.[78] Depending on the receptor concentration, virions slide within the membrane or roll over the membrane, after which they are arrested in confinement zones defined by the cortical actin network and eventually internalized.[78,136,268] Reoviruses do not show lateral motion after binding.[73] In the case of a bunyavirus, Uukuniemi virus, the rapid clustering of receptor molecules (GFP-tagged DC-SIGN) can be seen to occur at the site of virus binding.[151]

Filopodia have been shown to play an active role by providing directed transport of surface-associated virus particles toward the cell body.[140] They are thin, mobile extensions of the plasma membrane stabilized by an actin filament bundle. Such “virus surfing” occurs at a rate of 1-2 mm/min, mirroring the rate of retrograde actin flow from the tip of the filopodia inward.[233] It is actin dependent and inhibited by inhibitors of myosin II. Although such motility of viruses is not essential for infection in tissue culture cells, it may play a role in tissues.

After endocytosis, the actin- and microtubule-dependent movement of intracellular vacuoles, viruses, and naked capsids inside the cell can also be visualized.[73,107,139,228,283] This is illustrated by fluorescent influenza A viruses, which after a slow period of actin-restricted motion in the cell periphery undergo rapid microtubule-mediated transport toward the perinuclear space where penetration by membrane fusion occurs.[139,228] In the case of adenovirus 2, the transport of capsids along microtubules is both plus- and minus-end directed, but net transport in the minus-end direction allows the virus to reach the nucleus.[266] The entry of parvovirus adeno-associated virus 2 has been traced all the way to the nucleus, inside of which it moves unidirectionally along well-defined pathways.[239]

Virus-Induced Signals

Many viruses use the host cell’s signaling systems to promote entry and optimize infection.[97,177,186] Viruses take advantage of the fact that cells are exquisitely sensitive to ligands that bind to the plasma membrane, particularly if they induce clustering of surface components. More specifically, signaling is used to trigger access to coreceptors, to induce endocytic responses, to reprogram endocytic pathways, and to induce favorable intracellular conditions for infection.

Signaling starts at the plasma membrane after binding of the virus to receptors and formation of receptor clusters. Depending on the virus, receptors, and host cells, initial binding can lead to activation of tyrosine or other kinases, which, in turn, trigger cascades of downstream responses at the plasma membrane, in the cytoplasm, and, in some cases, in the nucleus. Virus-induced signaling depends on the usual panel of second messengers (phosphatidylinositides, diacylglycerides, and calcium), and on numerous regulators of membrane trafficking and actin dynamics.

One well-studied case that demonstrates the complexity of virus-induced signaling is provided by adenoviruses 2 and 5, which use CAR and integrin $\alpha v\beta 3$ as receptors.[97,186] Endocytic internalization occurs via clathrin-coated vesicles, and penetration takes place in endosomes. The interaction with the integrin triggers activation of p85/p110, a PI(3) kinase. The synthesis of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ activates protein kinase C. Small GTPases (eg, Rab and Rho family members) are also activated. One of the downstream responses is the transient activation of macropinocytosis, an actin-dependent process that results in a rapid increase in internalization of fluid.[169] This response seems to promote subsequent penetration of adenovirus from endocytic vacuoles by virus-induced rupture.

Another example is SV40, which is entirely dependent on signaling for entry. After binding to GM1 gangliosides, the virus induces local activation of tyrosine kinases, which results in actin filament reorganization, activation of caveolar dynamics, internalization of the virus in caveolar or lipid raft vesicles, and induction of long-distance transport of the virus-containing vesicles.[60,191,203,257] More than 50 different kinases were shown to regulate the entry and early steps in the infection of HeLa cells by this virus.[202]

A final example involves Kaposi sarcoma–associated herpesvirus (human herpesvirus 8).[41] The glycoprotein gB of this virus possesses an RGD sequence in the ectodomain that allows it to bind to the integrin $\alpha 3\beta 1$. Binding activates focal adhesion kinase (FAK) and Src kinases, which, in turn, activate PI(3) kinases and Rho GTPases. Furthermore, via the PI(3)K-PKCzeta-mitogen activated or extracellular regulated kinase (MEK) pathway, the virus induces the extracellular signal-regulated kinase 1 and 2 (ERK1/2). Activation of these pathways leads to major alterations in the actin cytoskeleton, and the virus is internalized by macropinocytosis in human fibroblasts.

Endocytic Pathways of Infection

Whether viruses penetrate into the cytosol directly through the plasma membrane or after endocytosis has been a hotly debated issue from the beginning of animal virology. It is now recognized that a majority of animal viruses—whether enveloped or nonenveloped—make use of endocytosis for productive infection. They exploit one or more of several endocytic mechanisms offered by cells (Fig. 4.2), and most of them enter endocytic vacuoles where penetration into the cytosol occurs often triggered by low pH. Enveloped virus families (eg, paramyxo-, herpes-, and retroviruses) that can penetrate directly through the plasma membrane because they do not require endocytosis for fusion may still depend

on endocytosis for productive infection at least in some cell types.[134,188] Here, the reason may be that fusion at the plasma membrane remains nonproductive because it does not ensure passage of the capsids through further barriers such as the actomyosin cortex.[158]

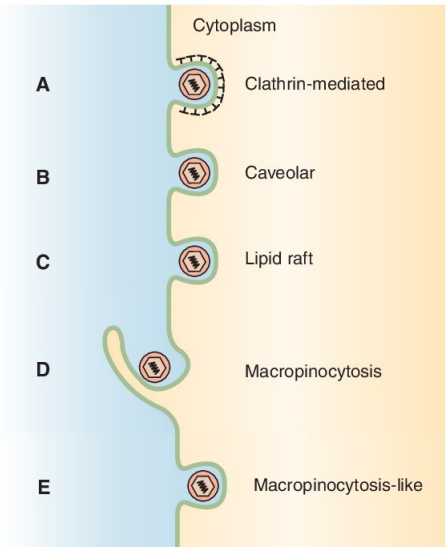

FIGURE 4.2 **Mechanisms of endocytosis used for virus entry**. Viruses can use different mechanisms of endocytosis. The majority of animal viruses enter cells by endocytosis. The mechanisms include **(A)** clathrin-mediated endocytosis, the most commonly used mechanism for virus entry; **(B)** caveolar endocytosis, a caveolin and lipid raft–dependent mechanism mainly used by polyomaviruses; **(C)** a caveolin-independent, lipid raft–mediated mechanisms with many similarities to the caveolar mechanism; **(D)** macropinocytosis, a transient, ligand-induced, actin-dependent mechanism; and **(E)** various macropinocytosis-like mechanisms which are caveolin and clathrin independent.

The main reason why endocytosis is a preferred mode of entry is most likely that endocytic vesicles offer viruses a free ride through the cortical cytoskeleton and other barriers that encumber movement of virus-sized particles in the cytoplasm. By delaying their penetration, viruses can in this way get a ride to the perinuclear region of the cell. In endocytic vacuoles, viruses can, moreover, count on receiving specific cues such as a drop in pH and exposure to proteases to trigger penetration and uncoating. Because no trace of the virus is left exposed on the plasma membrane, immunorecognition of infected cell is delayed. Moreover, for nonenveloped viruses that use membrane lysis or pore formation for penetration, it may be essential to cross membranes of intracellular organelles to minimize damage to the cell.

In recent years, the landscape of endocytosis research has expanded dramatically beyond phagocytosis and the classic clathrin-mediated endocytosis pathway. New mechanisms include caveolar or lipid raft-mediated endocytosis, macropinocytosis, and several other macropinocytosis-like pathways that operate independent of clathrin and caveolae (Fig. 4.2).[67,166,175,199,278] The situation is often confusing because the mode of uptake of a virus can vary between cell types and strains, and many viruses can make use of multiple receptors and parallel routes of endocytosis in the same cell. In addition to caveolae, SV40 can, for example, utilize a related, noncaveolar pathway.[60] Influenza A uses both clathrin-mediated and clathrin-independent pathways,[162,228,244] and HIV-1, which can fuse with the plasma membrane, can in some cell lines also make efficient use of an endocytic pathway for entry.[57]

The use of multiple entry pathways poses challenges for antiviral drug design and repurposing. For example, chloroquine, an antimalaria drug that acts by raising endosomal pH, is known to inhibit viruses that enter cells by low pH-dependent endocytosis[232] (Table 4.2). In 2005, chloroquine was shown to inhibit SARS coronavirus (SARS-CoV) infection in primate-derived kidney cells (Vero).[282] Early during the COVID-19 pandemic, SARS-CoV-2 was shown to use ACE2 as an entry receptor, and chloroquine shown to inhibit its infection in Vero cells.[112,286] Shortly thereafter, chloroquine and its analog hydroxychloroquine were repurposed for treatment of COVID-19, despite efficacy concerns.[83] When the inhibitory capacity of chloroquine and hydroxychloroquine was tested in human lung cells (Calu-3), neither could prevent entry nor infection by SARS-CoV-2.[113] Direct comparison of SARS-CoV-2 entry indicated that the virus enters Vero cells by chloroquine sensitive, low pH-dependent endocytosis, and human lung cells through chloroquine insensitive, plasma membrane fusion.[197] Which of these entry pathways SARS-CoV-2 takes depends on the transmembrane serine protease, TMPRSS2 (reviewed in Ref.[118]). When present at the plasma membrane of target cells, TMPRSS2 cleaves the viral spike protein exposing the fusion peptide, which then initiates virion fusion at the cell surface. In the absence of TMPRSS2 cell surface expression, SARS-CoV-2 virions are taken up by endocytosis and spike cleaved by cathepsin L within endosomes resulting in fusion from the endosomal compartment.[34]

TABLE 4.2 pH dependence of virus families

Low pH Dependent	pH Independent
Adeno	Corona (majority)
Alpha Borna	Retroviruses (majority) Herpes (majority)
Bunya	Paramyxo
Corona (some)	Hepadna
Filo	Pox (some)
Flavi	Rota
Orthomyxo	Picorna (most)
Parvo	Noro
Papilloma Picorna (some) Pesti Pox (some) Rhabdo Arena Arteri Hepaci	

The use of cell type–dependent entry mechanisms likely explains the lack of *in vivo* efficacy of chloroquine and hydroxychloroquine against SARS-CoV-2 seen in large, randomized trials.[25,93] This example serves to highlight that the use of multiple receptors, redundant endocytic pathways and different modes of entry provides viruses with a degree of flexibility and adaptability that makes entry a difficult step for host organisms to protect themselves against.

The cell biology of endocytosis and its regulation is complex.[49,145] High-throughput siRNA silencing, haploid genetic, and CRISPR screens have led to the identification of host genes critical for early infection of tissue culture cells including essential attachment factors, entry receptors, and proteins required for distinct endocytic trafficking pathways.[45,110,202,216] Entry studies are often further complicated by the fact that only a small fraction of the cell-associated viruses enter productively. Because most morphological and biochemical methods fail to distinguish between particles that enter productively and those that do not, studies using these methods must be complemented with readouts based on infection (ie, the biological outcome of successful entry). This involves the use of inhibitors, dominant negative mutants, small interfering RNAs, mutant viruses, and mutant cell lines. Only a combination of methods allows pathways of productive entry to be charted with confidence.

Clathrin-Mediated Endocytosis

The clathrin-mediated endocytic pathway is used by many viruses (Figs. 4.2 and 4.3). It is a process that cells use to internalize a spectrum of receptor-bound ligands, fluid, membrane proteins, and lipids for recycling or degradation. By binding to receptors that have the internalization signals necessary for inclusion in clathrin-coated pits, viruses make use of this pathway as opportunistic ligands. Uptake is characterized by rapid kinetics (viruses are generally internalized within a few minutes after binding) and by high capacity (3000 virus particles or more per minute).[159] With a diameter up to 120 nm, coated vesicles are large enough for the endocytosis of most animal viruses. Sometimes larger particles (eg, vesicular stomatitis virus [VSV]) can be accommodated.[163]

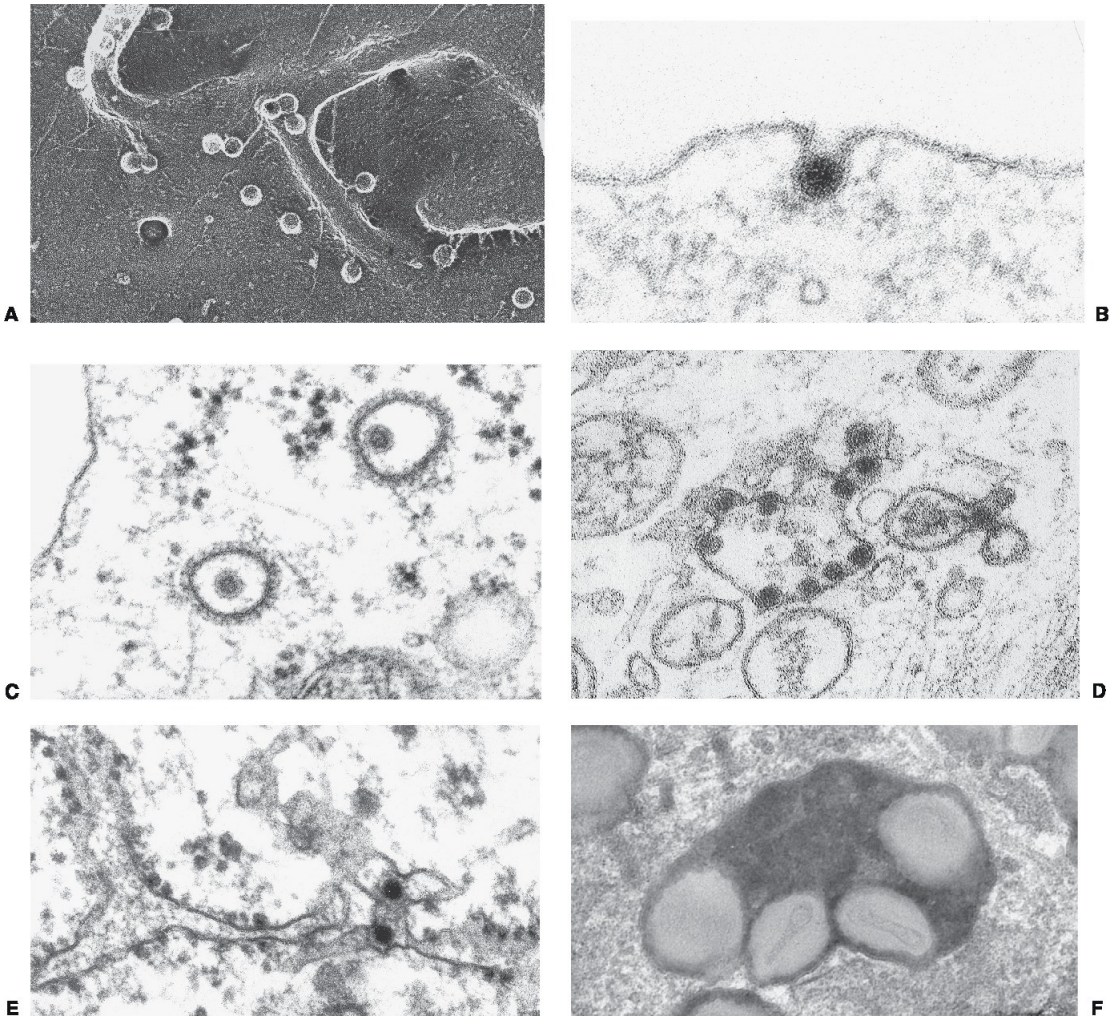

FIGURE 4.3 **Electron microscopy of virus endocytosis**. **A**. A surface replica of a BHK21 cell with Semliki Forest virus (SFV) particles attached. Some particles are bound to microvilli, and one is about to be endocytosed inside a coated vesicle. (Courtesy of John Heuser and Ari Helenius.) **B**. Internalization of a SV40 particle by caveolar- or raft-mediated endocytosis. The tight fitting vesicle in which the virus is internalized has a diameter of about 60-70 nm, and it has no visible coat. (Courtesy of Jürgen Kartenbeck and Ari Helenius.) **C**. SFV particles in clathrin-coated vesicles. (Adapted with permission of Rockefeller University Press from Helenius A, Kartenbeck J, Simons K, et al. On the entry of Semliki forest virus into BHK-21 cells. *J Cell Biol*. 1980;84(2):404-420; permission conveyed through Copyright Clearance Center, Inc.) **D**. SV40 particles in an early endosome. **E**. Incoming SV40 particles in a smooth membrane section of the endoplasmic reticulum, which they reach via the endocytic pathway. (Adapted with permission of Rockefeller University Press from Kartenbeck J, Stukenbrok H, Helenius A. Endocytosis of simian virus 40 into the endoplasmic reticulum. *J Cell Biol*. 1989;109(6 Pt1):2721-2729; permission conveyed through Copyright Clearance Center, Inc.) **F**. Multiple vaccinia virus particles in a late macropinosome. Macropinosomes are stained with HRP for clarity. (Courtesy of L. Pokorny, J. Burden, and J. Mercer.)

Although clathrin-mediated endocytosis is a continuously ongoing process, it is under stringent control. Interestingly, when the uptake of VSV, influenza, and reovirus particles has been followed in live cells, it has been observed that most are internalized by clathrin-coated pits that form *de novo* under the virus particles.[56,121,228] Only a few enter via preexisting clathrin-coated pits. Exactly how the virus induces a transbilayer signal to direct the assembly of the clathrin coat remains to be defined.

A role for clathrin-coated pits in internalization and infection can be demonstrated by inhibiting clathrin function using dominant negative mutants or depletion of adaptors such as epsin, eps15, AP2, or the clathrin chains themselves.[51,244] Inhibition of dynamin 2, a scission factor in clathrin vesicle formation, is not a sufficient indicator for clathrin involvement because dynamin 2 is also involved in other forms of endocytosis.

Caveolar and Lipid Raft–Mediated Endocytosis

The caveolar and lipid raft–mediated pathways of endocytosis were first observed for SV40 and mouse polyomavirus[7,60,221,257] (Figs. 4.2 and 4.3). They are cholesterol dependent, tyrosine kinase activated, cargo induced, and involve small endocytic vesicles. The cholesterol dependence reflects a central role of lipid rafts. Three variants of caveolar or lipid raft endocytosis are currently recognized[138]: (1) endocytosis via classical caveolae, dynamin 2 dependent; (2) noncaveolar, lipid raft–mediated endocytosis, dynamin 2 dependent; and (3) noncaveolar, lipid raft–mediated endocytosis, dynamin independent.

Caveolae constitute 70-nm flask-shaped indentations that contain caveolins and cavins as major protein components and a membrane enriched in cholesterol and sphingolipids.[199,219] Most cell surface caveolae are stationary, with a minority population undergoing a local cycle of fission and fusion with the plasma membrane.[204,279] When local tyrosine phosphorylation is activated by a virus such as SV40, caveolae become more dynamic.[138,199] With a virus particle trapped inside, the caveolae pinch off and move into the cytoplasm where they fuse with endosomes.

SV40 and other polyomaviruses also enter in vesicles devoid of caveolar proteins.[60] After association with lipid rafts in the plasma membrane or artificial liposomes, the binding of SV40 to multiple receptor gangliosides, GM1, leads to the induction membrane curvature following the shape of the virus and the formation of tight-fitting indentations of variable depth.[77] For detachment of a vesicle, these inward-oriented, virus-containing "buds" require the activation of kinases and other cellular factors. The process shares many features with the endocytic mechanism triggered by certain bacterial toxins, such as Shiga and cholera.[224]

Macropinocytosis and Macropinocytosis-like Mechanisms

Among the clathrin-independent mechanisms, macropinocytosis and related processes are commonly used by larger viruses (Figs. 4.2 and 4.3). These include vaccinia, herpes, adeno 3, and Ebola virus but evidently also in some cases by smaller viruses such as HIV-1 and influenza A.[6,126,157,174,229] Macropinocytosis is ligand triggered, transient, actin dependent, and regulated by a complex signaling pathway.[175,267] The physiological cargo is mainly composed of extracellular fluid that is trapped in large vacuoles, the formation of which depends on plasma membrane ruffling. The process differs from phagocytosis in the signaling pathways used and in that it can be activated in most cell types, not only in specialized cells.[267] In addition, by serving as a major mechanism in the elimination of apoptotic debris in tissues, macropinocytosis differs from phagocytosis by failing to activate innate immune responses and inflammation.[173]

In macropinocytosis, the interaction of viruses with the plasma membrane induces a rapid activation of receptor tyrosine kinases or integrins. This leads to a signaling cascade that usually involves the activation of GTPases Rac1 or cdc42, the p21-activated kinase (PAK1), myosin II, and numerous other kinases and signaling factors.[175] A change in the dynamics of cortical actin leads to ruffling of the plasma membrane, where the ruffles can take the form of lamellipodia, filopodia, and blebs. In the case of vaccinia virus and Kaposi sarcoma virus, internalization by macropinocytosis occurs during bleb retraction, and the viruses enter macropinosomes from which they escape by membrane fusion.[174,277]

Viruses including HPV-16, AAV2, HCMV, and measles have been purported to undergo cell type–dependent macropinocytosis-like entry.[63,142,190,235] These macropinocytosis-like pathways can deviate from classical macropinocytosis with regard to cell signaling, membrane ruffling, GTPase activity, and increased fluid uptake. For example, the entry requirements for HPV-16 are typical of macropinocytosis, including Na^+/H^+ exchange, RTK/PI3K signaling, and actin polymerization. Yet, virus internalization occurs through small uncoated vesicles and is not associated with membrane ruffling or GTPase activation.[235] On the other hand, AAV2 entry induces membrane ruffling, requires Na^+/H^+ exchange and the GTPase CDC42, but does not increase fluid phase uptake.[190] As more is learned about the mechanisms underlying macropinocytosis, it is becoming increasingly clear that there is a complex spectrum of related pathways that vary on the general theme.[176]

The Endocytic Pathway

The early endosomes in the periphery of the cytoplasm receive incoming viruses a few minutes after internalization (Figs. 4.4 and 4.5). Early endosomes constitute complex, heterogeneous organelles with tubular and vacuolar elements. They are mildly acidic (pH 6.6-6.0), which is enough to activate viruses with a high pH threshold for penetration, and these viruses are thought to penetrate from early endosomes.[160] Most viruses are not activated in this pH range and continue deeper into the degradative arm of the endocytic pathway in order to penetrate from late endosomes or endolysosomes.

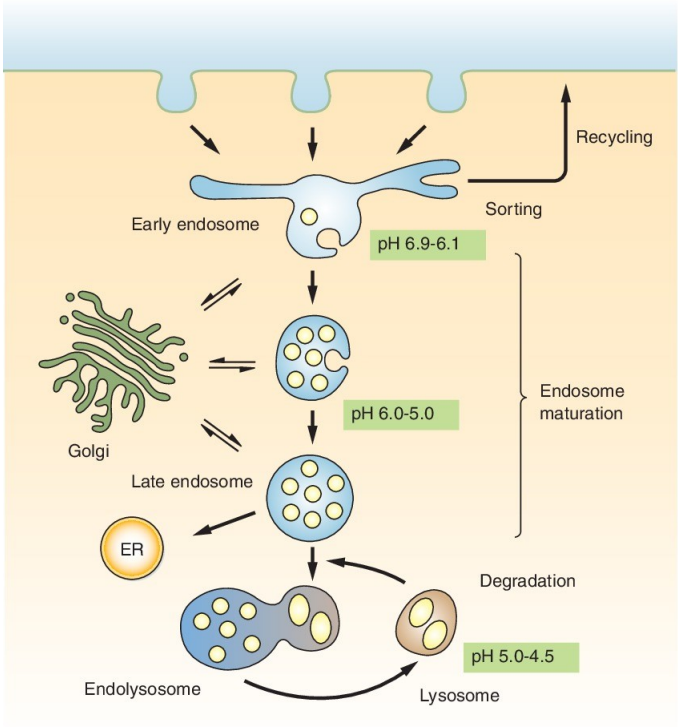

FIGURE 4.4 **The endosomal pathway**. The pathway functions as two interconnected cycles of membrane trafficking. One involves the plasma membrane, early endosomes, and a variety of carrier vesicles. Its major role is the sorting and recycling of incoming membrane components, ligands, and fluid via the endosome back to the cell surface. In this pathway, the pH does not drop below about 6.0, and the cargo is not exposed to a spectrum of lysosomal enzymes. The main function of the second cycle, the lysosome cycle, is degradative (ie, the

down-regulation of receptor-ligand complexes, degradation, and processing of incoming nutrients and their carriers, digestion of autophagic substrates, elimination of incoming pathogens, etc.). The endocytic cargo to be degraded, including viruses, is sorted from early endosomes into late endosomes, and these deliver the cargo to lysosomes 10-40 minutes after formation by fusing with them to form endolysosomes. The late endosomes undergo a complex maturation process, acquire intraluminal vesicles, and move along microtubules to the perinuclear region of the cells. Degradation occurs in the endolysosomes through the action of soluble hydrolases. Endolysosomes and lysosomes keep fusing with new late endosomes in a continuous cycle. Early and late endosomes communicate via vesicle trafficking with the Golgi complex, and late endosomes and endolysosomes have a poorly understood connection to the endoplasmic reticulum.

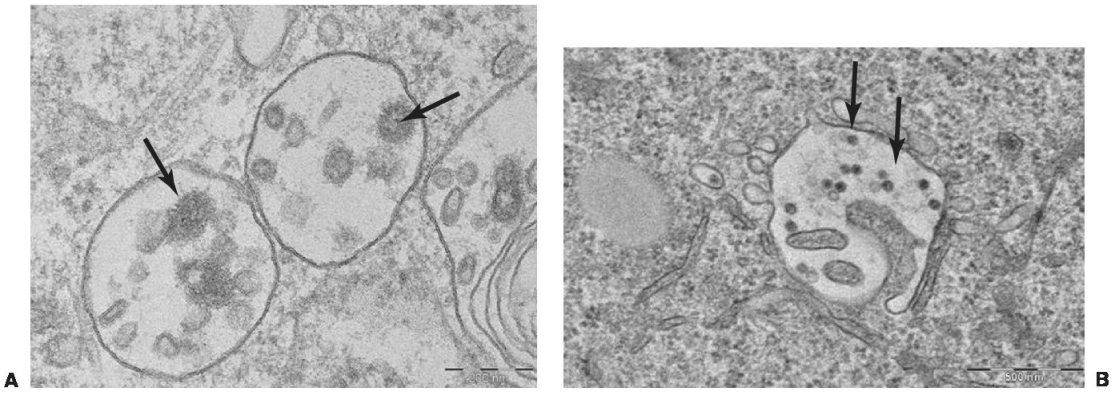

FIGURE 4.5 **Viruses enter endosomes**. Endosomes are cytoplasmic vacuoles with complex and quite heterogeneous morphology. Thin section electron microscopy reveals that many of them are filled with intracellular vesicles and membrane lamellae. **A**. Influenza A viruses (*arrows*) are here seen in multivesicular endosomes closely connected to microtubules. **B**. Human papilloma-16 pseudovirus particles are here seen in an endosome with tubular extensions. (Courtesy of Roberta Mancini.)

To understand virus entry, it is important to understand the cell biology of endosomes. There are numerous reviews that provide insights into this important topic.[26,64,102,171,173,230,237] One of the central topics is the maturation of late endosomes, a program of changes that prepares them for fusion with lysosomes.[115] The changes include a further drop in pH, a switch of predominant Rabs from Rab5 to Rab7, a switch from phosphatidylinositides (PI(3)P-PI(3,5)P_2), exchange of tethering factors for fusion, exchange of microtubule-dependent motors, formation of intralumenal vesicles, and accumulation of lysosomal membrane proteins and hydrolases. It is a complex process where the various alterations are coordinated and interdependent. The key factors include small GTPases of the Rab, Arf, and Rho families and their effectors, phosphatidylinositides and their kinases and phosphatases, protein ubiquitination and the endosomal sorting complex required for transport machinery responsible for the formation of intralumenal vesicles in endosomes, the vacuolar ATPase responsible for acidification, and various soluble *N*-ethylmaleimide–sensitive factor attachment protein receptors and tethering factors required for selective fusion events.

Late penetrating viruses such as influenza virus, minor group rhinoviruses, polyomaviruses, and bunyaviruses depend on a smoothly functioning maturation program. They require the formation of late endosomes, the reduction in pH, and transport of the endosome to the perinuclear region.[26,101,150,243] Infection can be blocked by interfering with the maturation program using inhibitors, dominant negative mutants, and siRNA depletion of endocytosis factors.

The significance of low pH in endosomes as a cue for the activation of virus penetration was discovered a long time ago.[106] It is now clear that for the majority of animal viruses, low pH is needed to trigger conformational changes in metastable viral particles and fusion proteins, thus activating membrane penetration mechanisms (see Chapter 3). Viruses with a relatively high pH threshold (pH 6.5-6.0) such as VSV are activated 3-10 minutes after internalization in early endosomes.[159] Viruses with a lower pH threshold are sorted from early endosomes into the degradative branch and penetrate later (10-50 minutes or even longer after infection) and less synchronously in late endosomes or endolysosomes that have a pH of 6.0-4.9. For example, influenza A virus, with a pH threshold of 5.6-4.9, passes via early endosomes to perinuclear late endosomes before membrane fusion occurs.[245]

Macropinosomes, the intracellular carrier vesicles formed during macropinocytosis, undergo maturation in a fashion similar to endosomes.[31,66,176] This includes acidification, a switch in Rab and phosphatidylinositide identity, and ultimately fusion with lysosomes. Unlike primary endocytic vacuoles, once formed macropinosomes do not feed into early endosomes but undergo maturation, tubulation, and recycling themselves. For this, macropinosomes rely on Rab34 and sorting nexins in addition to the classical endosomal markers.[128,265] Viruses that rely on macropinosome maturation for penetration include Ebola, HHV8, and vaccinia.[176] For vaccinia, which has a pH threshold of 4.0-5.5, macropinosome maturation, culminating in delivery of virions to late endosomal/lysosomal compartments is critical for membrane fusion and penetration.[222]

In exceptional cases, such as some of the nonenveloped polyomaviruses, viruses continue even farther, moving from late endosomes to the endoplasmic reticulum (ER) where penetration occurs through the ER membrane.[44,74,213] While this pathway is not completely understood, after uptake polyomavirus-containing late endosomes are thought to be guided to the ER through initial receptor interactions (murine polyomavirus and ganglioside GD1a),[95,275] or recruitment of cytosolic factors (BK polyomavirus interaction with Rab18).[305] Recent evidence indicates that upon arrival at the ER, two ER membrane protein complex subunits, EMC 4 and 7, facilitate docking of polyomavirus-containing late endosomes.[9] Through interactions with late endosome-associated Rab7 and ER resident protein syntaxin18, EMC 4 and 7 are thought to stabilize late endosome-ER contact allowing for transport of polyomaviruses between these two compartments.[275,305]

Penetration by Membrane Fusion

The membrane of an enveloped virus is a *de facto* transport vesicle designed for intercellular membrane traffic (Fig. 4.6). Like intracellular transport vesicles, the transport process relies on budding, fission, and fusion. The cargo is the viral capsid, which does not have to cross the hydrophobic barrier of a membrane. The fusion reaction during entry can occur with the plasma membrane or with the limiting membrane of an

endosome. On the basis of studies with VSV, it has been proposed that a virus can also fuse with lumenal membrane vesicles inside multivesicular endosomes followed by a delayed second fusion event between the vesicle and the limiting membrane of late endosomes.[101,226] The second fusion would have to depend on a cellular rather than viral fusion machinery.

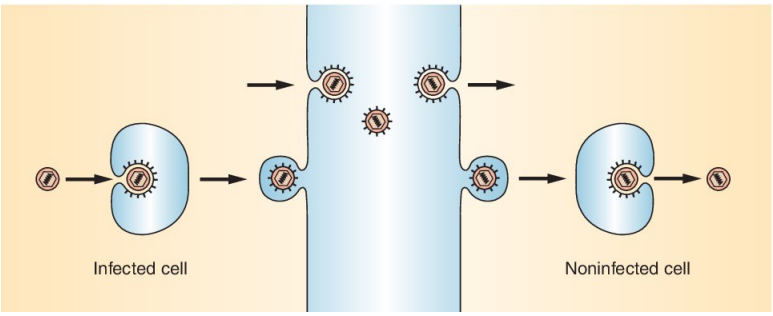

FIGURE 4.6 **Enveloped viruses use a vesicle shuttle mechanism**. They transport the capsids and the viral genome from cell to cell using a vesicle transport strategy based on membrane fission and fusion. The viral envelope constitutes the transport vesicle, and the capsid is the cargo. The vesicle is formed after capsid loading and glycoprotein sorting in the infected cell by budding and membrane fission at the plasma membrane or internal membranes. The viral envelope membrane protects the capsid during the voyage through the extracellular space. After associating with a new host cell, the virus delivers the capsid into the cytosol by membrane fusion, either at the plasma membrane or after endocytosis, at the limiting membranes of intracellular organelles. The advantage of this strategy is the viral genome, and accessory proteins can be transferred from cytosol to cytosol without the need of translocation directly across the hydrophobic barrier of any membrane.

As described in Chapter 3, viral fusion proteins are integral membrane proteins, with the bulk of their mass external to the viral envelope. They are usually glycoproteins and occur as homo- or hetero-oligomers. Many of them combine fusion and receptor-binding activities in the same molecule. To become fusion competent after folding and assembly in the ER, they are often primed by proteolytic cleavage during transit through the trans-Golgi network (TGN). Typically, the proteases responsible for priming are furin family convertases that cleave either the chains involved in fusion directly (ie, in myxo-, retro-, and paramyxoviruses) or the companion proteins in the spike protein complex (ie, alpha- and flaviviruses).[132,256,287] If virus assembly and budding occurs intracellularly, as for flaviviruses, the activating cleavages take place as the assembled viral particles pass through the TGN.[105] In some cases, such as Ebola virus and SARS-CoV-2, proteolytic activation can occur as part of the entry program by proteases present in endocytic vacuoles.[43,112]

Proteolytic priming renders fusion proteins metastable and, thus, competent to undergo large, irreversible conformational changes without added energy.[40] This is important because, to elicit fusion, they must undergo changes that dramatically alter their quaternary or tertiary structure. As a result, they expose previously hidden hydrophobic peptide segments (fusion peptides) that interact with the target membrane, and in doing so allow the proteins to be hydrophobically anchored in both membranes.[289] The conformational change is thought to provide the energy required to overcome the so-called *hydration force* that prevents biological membranes from fusing spontaneously.[217]

The changes in conformation are triggered either by low pH in endosomes or by interaction between viral proteins and receptors in the plasma membrane. Sometimes receptor binding followed by low pH is needed. This type of receptor-mediated priming, observed for some retroviruses,[183] may allow the virus particle to limit conversion of spike glycoproteins to the site most intimately in contact with the target membrane. The priming and activation of viral membrane fusion proteins as well as the mechanisms of fusion are discussed in greater detail in Chapter 3.

Penetration by Nonenveloped Viruses

Nonenveloped viruses penetrate into the cytosol through the limiting membranes of internal organelles (eg, endosomes, lysosomes, the ER). The cues that trigger penetration are similar to those used by enveloped viruses (eg, receptor-binding, low pH, redox environment). These viruses must, however, transfer their capsids, genomes, and associated proteins through a cellular membrane without the convenience of the membrane-fusion mechanism.

The mechanisms involved have proved challenging and remain incompletely understood. It seems, however, that nonenveloped viruses can use three general strategies:

a. *Membrane puncture*. The virus particle generates a pore in the membrane through which the genome is selectively released into the cytosol. The viral capsid does not enter the cytosol, and release of fluid phase markers from the lumen of the organelle is either undetectable or limited to small molecular weight compounds.

b. *Perforation*. The entire capsid is transferred through the membrane without major lysis of the membrane and little loss of lumenal fluid markers.

c. *Lysis*. The virus particles induce breakage of the membrane of cytoplasmic organelles, allowing the virus and other lumenal contents to be released into the cytosol.

The puncture or pore mechanism is favored for picornaviruses.[89,114] Some rhinoviruses and foot and mouth disease virus are acid activated and penetrate from endosomes, whereas others (eg, polio, coxsackie B, and echovirus 1) are pH independent but penetrate from endosomes or other intracellular organelles. In the case of poliovirus, which is one of the best characterized, penetration is induced by binding of a cluster of poliovirus receptor (PVR) molecules to “canyons” in the capsid surface. This triggers a large, concerted, irreversible change in the particle, the so-called *eclipse*, which leads to the formation of a penetration competent conformation. An internal protein, VP4, is released, and the myristylated *N*-terminus of VP1 inserts into the endosomal membrane.[27,87] The RNA is most likely released to the cytosolic side of the membrane through a narrow pore.[273] According to this view, penetration and uncoating occur simultaneously, and the capsid does not enter the

cytosol. Cryo-electron microscopy (cryo-EM) studies have shown that one of the icosahedral vertices interacts with five receptors that connect the modified particle intimately with the membrane.[30]

For viruses that use a perforation strategy, escape of polyomaviruses from the ER into the cytosol is mechanistically the best understood[218] (reviewed in Ref.[44]). In the case of SV40, upon arriving in the ER resident redox proteins PDI, ERp57 and ERdj5 reduce and isomerize virion disulfides.[234] This causes the disassociation of the 12 vertex pentamers composed of the major capsid protein VP1. This partial disassembly exposes minor capsid proteins VP2 and VP3.[61,192] The hydrophobic, myristylated N-terminus of VP2 inserts into the ER membrane where it is proposed to initiate the formation of a penetration "foci" through recruitment of ER proteins Bap31/Bap29 and the luminal chaperone BiP.[91,96] Accumulation of DNA J proteins to foci serves to recruit a cytosolic chaperon complex composed of Hsc70, Hsc105, SGTA, and Bag2 that facilitates the escape of the modified particle into the cytosol.[68-74,284]

Recently, cryo-EM and cryo-electron tomography (cyro-ET) studies of bluetongue virus (BTV) and rhesus rotavirus (RRV) have provided the most compelling images of the perforation mechanism to date.[108,296] BTV and RRV are each composed of three capsid layers: outer, intermediate, and inner. Both viruses are endocytosed where upon changes in endosomal conditions (pH in the case of BTV and CA^{2+} in the case of RRV) trigger the release of outer capsid proteins and subsequent conformational "activation" of the respective penetration proteins. This causes them to project from the surface of the virus facilitating their engagement with the target membranes. While the details remain unknown, this is presumably followed by fusion pore formation and delivery of intact double-capsid layer particles into the cytosol. For BTV, cryo-ET data support the opening of a single fusion pore, which expands to accommodate the double-layer particle.[296]

For parvoviruses, evidence is accumulating that the *N*-terminal domain of VP1 possesses a phospholipase 2 domain activated by low pH.[53,104] It is likely that this promotes membrane penetration of the intact virus by modifying the permeability of endosomal and lysosomal membranes. Lysis of the membrane is not detected.

Adenoviruses make use of a lytic mechanism.[240] The best studied are adenoviruses 2 and 5, which penetrate by acid-activated rupture of the endosomal membrane.[167,170] The lytic effect is thought to involve a change in the penton base and exposure of an amphipathic helix in protein VI, but the mechanism is unclear.[185] Altogether, the process is complex; it depends on low pH, the integrin receptors, cleavages in structural proteins induced by the L3/p23 viral protease, the release of fiber proteins, activation of macropinocytosis, and signaling through protein kinase C.[167,170,186]

Intracellular Trafficking

Before they can replicate, viruses and capsids delivered into the cytosol must be transported to the correct location before uncoating and replication can take place. Within the nucleus, replication usually occurs in defined foci. In the cytosol, it is often associated with specific membrane organelles (eg, the ER or the ER-Golgi intermediate compartment) or with virus factories in the perinuclear space.[193,294]

Given the extreme crowding in the cytoplasm that prevents diffusion of virus- and capsid-sized particles as well as uncondensed forms of DNA or RNA, it is not surprising that viruses rely on cytoplasmic transport systems offered by the cell.[65,99,154,251] For long-distance transport, viruses mainly exploit microtubule-mediated mechanisms. When actin filaments play a role, it is usually in short-distance movement close to the plasma membrane. Although viruses can undergo partial disassembly *in transit* through the cytosol, they postpone final uncoating of the condensed genome until they have reached their final destination. Viruses travel variable distances. To reach the cell body, neurotropic viruses that enter via axons may have to move in a retrograde direction over the full length of axons, which can be more than 1 m in length.

To move through the cytoplasm, incoming viruses have two options. They can postpone penetration into the cytosol and move as cargo in endocytic vesicles and thus benefit from the motor-driven transport of vesicles and organelles through the cytoplasm. Alternatively, they can penetrate early into the cytosol, in which case the viruses or their capsids are themselves responsible for associating with molecular motors and adaptors (eg, dynein and kinesins). The former strategy is used by viruses that enter by endocytosis, the latter by viruses that prefer to penetrate through the plasma membrane. Many viruses make use of both; part of the journey is mediated by vesicular traffic, the rest by cytosolic transport.

An exception to this is HPV, which engages cytoplasmic factors despite remaining within membrane vesicles during its entire journey from the cell surface to the nucleus (reviewed in Ref.[297]). After HPV internalization into endocytic compartments, virions are subverted to the retrograde pathway and are trafficked to the TGN, Golgi, and ER.[62,79,80] To reach these compartments, the viral protein L2 engages retromer, a cytoplasmic protein complex that selectively delivers endosomal cargo to the TGN.[238] The C-terminus of L2 contains a cell penetrating peptide within a membrane destabilizing sequence and two retromer binding sites.[210,297,302] Elegant split GFP assays showed that early during HPV entry, the cell penetrating peptide in L2 directs protrusion of the C-terminus through the endosome membrane exposing the retromer binding sites to the cytoplasm.[302] L2 then engages retromer and other cytoplasmic proteins involved in entry.[16,207] Consistent with this mechanism, L2 CPP and retromer binding site mutants, as well as depletion of retromer from target cells prevent retrograde trafficking of HPV resulting in accumulation of virions in endosomes.[146,210,302]

Viruses that enter by endocytosis then penetrate into the cytosol (eg, adeno- and parvoviruses) tend to use microtubule-mediated transport to bring capsids toward the cell center.[239,264,266] Although transport of capsids along microtubules is often bidirectional and characterized by stops, restarts, and changes in direction, net transport generally occurs in the minus-end direction toward the microtubule-organizing center (MTOC), where viruses and capsids are often found to accumulate before transport to the nucleus.[215] Whether they switch to plus-end directed motors for the final leg of transport is not known, with the exception of adenovirus. Upon reaching the MTOC, adenovirus bidirectional microtubule motility slows, and virions detach from microtubules.[285] The nuclear export protein CRM1 was found to be required for both processes, as well as association of adenoviruses with nuclear pores and genome delivery.[262,285] It is though that CRM1 may establish a perinuclear spatial gradient that serves to guide microtubule-independent movement of adenovirus from the MTOC to nuclear pores.

Priming the Uncoating Program

Penetration brings many viruses and viral capsids for the second time into a cytosolic environment. The first time is when they assemble in the

cytosol of an infected cell or when they pass through the cytosol on their way from the nucleus to the extracellular space. During entry into a new host cell, the agenda involves disassembly and uncoating instead of assembly. This means that, in the entry phase, something must be profoundly different either about the virus itself or the cell.

Usually, the difference is in the virus or the capsid because it has undergone structural alterations *in transit*. After release from the infected cell or during earlier stages of entry, the viruses are structurally *reset* so they can respond to cellular cues according to requirements of the uncoating program. The best illustration of this is provided by retroviruses, in which the viral protease induces a series of cleavages in Gag and Gag-Pol proteins during and after virus budding. The capsid is reorganized and ready for reverse transcription and for the formation of functional preintegration complexes (PICs) in the cytosol of a new target cell.[189]

Another example is influenza A, in which the switch involves a change in the properties of the matrix protein (M1). M1 serves as an adaptor between the virus ribonucleoproteins (vRNPs) and the viral membrane as well as between the vRNPs, and it plays a crucial role during assembly of these components during virus assembly and budding.[220,308] During entry, dissociation of these interactions is induced by an irreversible conformational change in M1 triggered by acid exposure in endosomes.[32] To acidify the internal space of the virus, where the M1 and the vRNPs are located, the viral membrane possesses acid-activated proton channels in the form of M2 protein complexes.[208,263] If the M2 proton channel is blocked using amantadine, a specific M2 channel blocker used as an anti-influenza drug; HA-mediated fusion occurs normally in endosomes, but the vRNP and M1 fail to dissociate from each other, and transport of vRNP to the nucleus is inhibited.[33,161]

Alphaviruses seem to use an altogether different strategy. Here, the *switch* seems to involve a change in the cell rather than in the viral capsid. A cellular factor required for uncoating of incoming capsids is inactivated during the course of infection, thus allowing assembly of progeny capsids. The factor in question is the 60S ribosomal subunit, which has high affinity binding sites for the viral capsid protein.[248,288] Incoming capsids rapidly lose capsid proteins to ribosomal subunits, and the viral RNA is thus liberated. When synthesis of structural protein starts later in an infection, newly synthesized capsid proteins bind to the ribosomal subunits and the ribosomal subunits can no longer interfere with assembly of progeny capsids.

Genome Uncoating

The entry of viruses includes partial or full disassembly as an essential, integrated part of the program. For enveloped viruses, uncoating involves loss of the envelope during membrane fusion. Often, the capsid thus released undergoes further stepwise uncoating steps. Once the capsids have reached the correct location within the cell, then, and only then, they release the replication competent form of the genome. In some cases (eg, retro-, reo-, and poxviruses), the cytosolic capsids serve as a protected site for reverse transcription of the genome or transcription of messengers prior to uncoating.[127,189] Poxviruses for example, undergo a particularly complex two-step uncoating process involving cellular and viral proteins.[130] Immediately upon cytoplasmic delivery, viral cores expand changing in shape from biconcave to oval.[58,236] This first “core activation” step correlates with the reduction of di-sulfide bonded core structural proteins, and the initiation of early gene transcription within the core.[147,236] The second uncoating step, genome release, is steadfastly dependent on viral early gene expression, suggesting that poxviruses time genome release through the expression of an early protein.[122,123] Using a virus-targeting RNAi screen, this “uncoating factor” was identified as the early poxvirus AAA+ ATPase D5, and it’s helicase activity deemed essential for uncoating.[131] In addition to D5, host proteasome activity was shown to be required for core breakdown and genome release.[178] While both D5 and proteasome activity is required, mechanistic understanding of their individual roles and interplay during poxvirus uncoating remains unknown.

Influenza A is another enveloped virus that takes advantage of host factors for uncoating. Beyond the M2-mediated priming step that serves to dissociate the vRNPs from M1, cellular aggresome components including free ubiquitin, HDAC6, and microtubule motors cooperate to physically disassemble viral capsids freeing the vRNPs, which are then shuttled into the nucleus.[10]

In the case of nonenveloped viruses, the uncoating process involves conformational changes, progressive loss of structural proteins, proteolytic cleavages, isomerization of intermolecular disulfide bonds, and weakening of intermolecular interactions.[98,114,167,187,234] For adenovirus 2, disassembly starts already at the cell surface with loss of some of the fibers followed by activation of a viral protease (the L3/p23 protease, located within the virion), proteolytic cleavage of capsid proteins, and loss of stabilizing capsid components. Final disassembly of the particle and DNA release occurs at the nuclear pore complex (NPC).

The conformational changes that accompany penetration and uncoating of polio and other picornaviruses have been extensively analyzed.[89,114,149] Depending on the virus, the initial uncoating event is triggered by receptor association, low pH, or both. Conversion from a 150S-160S particle to a slower sedimenting 135S particle occurs with elimination of the internal VP4 protein and externalization of the myristylated *N*-terminus of VP1. This leads to membrane association, followed by the release of the RNA, resulting in the RNA-free 80S particle. The single-stranded viral RNA is likely to escape through 1 of the 12 vertices, possibly aided by the VPg protein covalently linked to the 5′ end of the viral RNA.[29,129]

The capsids of viruses with a double-stranded RNA genome (eg, reoviruses) undergo many alterations in transit into the cell, but instead of releasing their genomic RNA in free form into the cytosol, they retain it in a modified capsid, which serves as an RNA replication and transcription factory.[42,246]

Nuclear Import

Most DNA viruses and a few negative-stranded RNA viruses replicate in the nucleus. To enter the nucleus, they can make use of the NPC for transport of the genome and accessory proteins into the nucleoplasm[48,55,98,290,291] (Fig. 4.7). Alternatively, the viruses may enter by rupturing the nuclear envelope, a process for which there is some evidence in the parvovirus field. These two entry routes allow infection of nondividing, terminally differentiated, interphase cells in which the nuclear envelope represents a permanent barrier. Finally, viruses and viral capsids may wait in the cytosol for the dissolution of the nuclear envelope during cell division. This mechanism, used by most retroviruses with the exception of lentiviruses and human papilloma viruses,[8,212] restricts infection to cell populations that undergo division. In principle, a fourth possibility would be penetration directly from the lumen of the ER through the inner nuclear membrane because the lumen of the ER is continuous with the space between the membranes in the nuclear envelope. Although some incoming viruses do pass through the ER and incoming viral particles have been occasionally seen between the two membranes of the nuclear envelope,[164] no evidence currently indicates that any viruses use this

pathway.

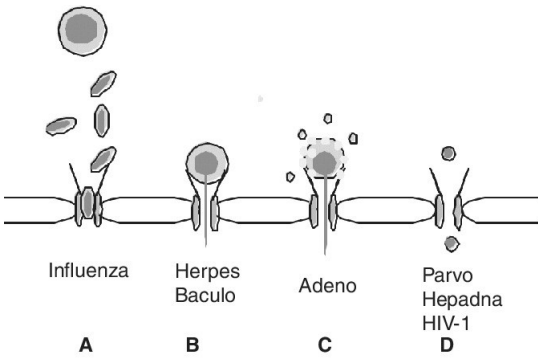

FIGURE 4.7 **Import of viruses and subviral particles through the nuclear pore complex**. To circumvent the size limitation (diameter 35-40 nm) of particle transport through the nuclear pore complex (NPC), viruses have evolved different strategies. **A**. The genome of a virus can be divided in multiple subgenomic particles with an elongated shape thin enough for individual entry (eg, influenza virus). **B**. Limited uncoating takes place in the cytosol with the generation of an opening in the capsid wall that allows the DNA to escape, leaving an empty capsid at the NPC (eg, herpes simplex virus 1). **C**. The virus dissembles after association with the NPC, allowing the genome and accessory proteins to pass through the NPC (eg, adenoviruses). **D**. The virus particles or capsids are small enough to enter as spherical particles without uncoating or major deformation with uncoating occurring in the nucleoplasm (eg, HIV-1).

Nuclear import via the NPC involves several steps: binding of import receptors, transport through the cytosol, association with the NPC, and transfer of the intact virus, a subviral complex, or a nucleic acid through the pore. To be recognized by the cellular import machinery, viruses and viral capsids make use of nuclear localization signals (NLSs) similar to those present on cellular proteins and ribonucleoprotein complexes. These signals in viral proteins are recognized by soluble receptor proteins (importins or karyopherins) that mediate recognition, transport, and docking of the viral capsids to the NPC. In some cases, the NLS and the importins involved have been identified. Exposure of the NLS is sometimes modulated by phosphorylation-induced conformational modifications to avoid premature capsid import in the infected cell,[125] and some viruses are thought to be processed by proteasomes.[281] It is also possible that viruses bind directly to the NPC without interaction with importins.

Binding of herpes simplex virus capsids to the NPC is mediated by capsid and tegument proteins.[195,200] After association with CAN/Nup214 and another NPC protein, hCG1, through the minor capsid protein pUL25, and after opening of the portal structure at one of the vertexes of the capsid, the viral DNA escapes into the nucleus, leaving an empty capsid behind at the mouth of the NPC (Fig. 4.8). Influenza A viruses deal with the problem of size limits by having a segmented genome. The eight subgenomic RNA are individually packaged into viral vRNP complexes. When interactions with the M1 protein are dissociated after exposure of the virus to low pH in endosomes, and the vRNPs are uncoated by HDAC6, they interact with importins, travel to the nucleus, and enter individually through the NPC (Fig. 4.7).[32,161,196] The vRNPs are rod shaped and, although variable in length, have a thickness of only 10-20 nm.[50]

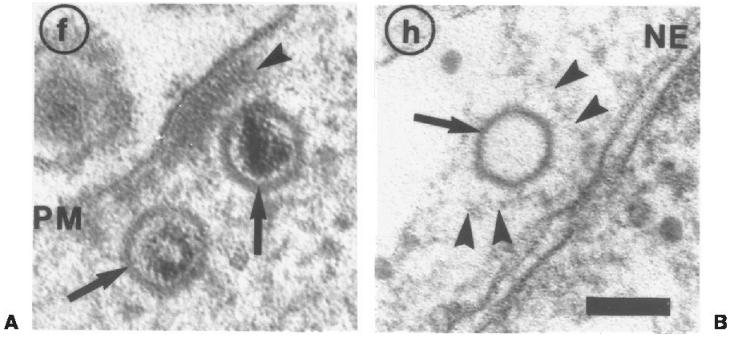

FIGURE 4.8 **Herpes simplex virus 1 (HSV-1) entry at the plasma membrane level and the nuclear envelope**. **A**. In HSV-1, virus can fuse with the plasma membrane and release the capsid and the tegument into the cytosol. A large part of the tegument can be seen separating from the capsid. **B**. After binding to the cytosolic fibers attached to the nuclear pore complex (NPC), the viral capsid releases its DNA genome through one of the pentameric facets, and an intact-looking empty capsid shell remains bound to the NPC for some time. PM, plasma membrane; NE, nuclear envelope. Space bar, 100 nm. (Courtesy of Beate Sodeik and Ari Helenius.)

Being too large, most viruses and capsids must undergo shape changes or disassembly before passage of the genome through the NPC. Partially uncoated and modified adenovirus 2 and 5 particles bind directly to the CAN/Nup214 nucleoporins on the outer surface of the NPC and to nuclear histone protein, H1.[274] For disassembly, NPC-bound capsids interact with the kinesin light chain Klc1/2, and through Nup358, the kinesin heavy chain Kif5c.[261] These interactions link viral capsids and NPCs to the kinesin-1 motor, which then physically disrupts and dislocates capsid fragments and Nups into the cytoplasm. This serves to free the linear, double-stranded viral DNA from the capsid and simultaneously disrupt the NPC providing the viral genomes access to the nucleus.[261]

The size limitation for transport through the NPC is an obvious problem. Although estimates of the functional pore diameter have been adjusted upward to 39 nm using purified nuclear envelopes,[198] only the smallest viruses and capsids are likely to enter intact without modifications. These include parvoviruses and the capsids of hepatitis B virus (HBV).[214,281] When nuclear import of injected HBV capsids through the NPC are imaged by electron microscopy, the capsids can be seen to line up on the cytosolic fibers of the NPC and inside the central channel (Fig. 4.9). Uncoating of these capsids occurs in the basket, a structure located on the nucleoplasmic side of the NPC.[214]

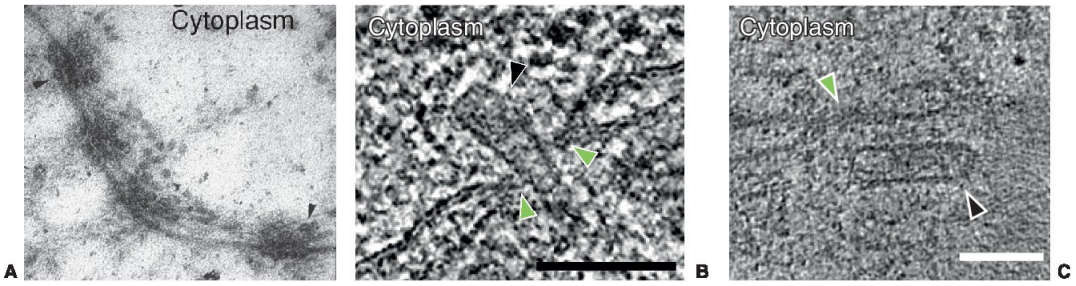

FIGURE 4.9 **Import of hepatitis B virus (HBV) and HIV-1 capsids through the nuclear pore complex (NPC)**. **A**. After injection into *Xenopus oocytes*, isolated HBV cores can be seen binding to fibers at the mouth of the NPC, and to line up in a row inside the channel of the pore. Uncoating of this capsid occurs in the basket on the nucleoplasmic side of the NPC. (Courtesy of Nelly Pante and Michael Kann.) **B**. A HIV-1 cone-shaped capsid (*black arrow head*) can be seen docked within the central channel of a NPC. *Green arrow heads* designate the edges of the NPC. **C**. A HIV-1 capsid that has lost its conical shape (*black arrow*) is seen in the nucleus of an infected cell. *Green arrow* designates the NPC. (**B and C** adapted from Zila V, Margiotta E, Turonová B, et al. Cone-shaped HIV-1 capsids are transported through intact nuclear pores. *Cell*. 2021;184(4):1032-1046.e18. Copyright © 2021 Elsevier, with permission.)

The PIC of retroviruses has been reported to be about 50-60 nm in diameter.[180] Too big to enter the nuclei without conformational adjustments, they remain in the cytosol until the breakdown of the nuclear envelope occurs during cell division.[141] Being capable of entering interphase nuclei, the PIC of HIV-1 and other lentiviruses are exceptions. The details of how and in which form lentivirus PICs are imported into the nucleus is emerging. Recent evidence gathered using 3D-correlative fluorescence light and electron microscopy (3D-CLEM) shows intact cone-shaped HIV-1 capsids docked to the cytosolic face of NPCs and within the central channel of NPCs (Fig. 4.9).[307] Supporting passage of capsids through the NPC, morphologically altered, open, empty capsid structures can be seen in the nucleus of infected cells (Fig. 4.9). Examination of NPC architecture in uninfected and HIV-infected cells by cryo-EM placed the diameter of the NPC central channel at ~64 nm, which is sufficient for the passage of 60 nm HIV-1 capsids.[307] While the HIV-1 capsids found in the central channel retain their cone shape and a hexagonal lattice —indistinguishable from capsids in mature virions—hyper-stable mutant HIV-1 capsids get stuck at the NPC.[103] This suggests that capsid flexibility or remodeling is needed for capsid passage through the NPC central channel and subsequent uncoating in the nucleus.

Transmission Directly From Cell to Cell

Discussion so far has focused on the mechanisms of entry by free viral particles attaching to the surface of cells. It is increasingly clear that there are situations in which infection occurs from cell to cell without participation of free virus particles[184,231] (Fig. 4.10). One mechanism involves fusion of an infected cell's plasma membrane with the membrane of a neighboring cell. In infected tissues and cultures, the result is the formation of multinucleated cells (ie, syncytia). Fusion in this case is mediated by viral fusion proteins expressed on the surface of the infected cell with receptors present on the noninfected cell. Commonly observed with paramyxovirus, herpesvirus, and other viruses with pH-independent fusion proteins, cell fusion provides a mechanism for transmitting infection independently of virus particle assembly.[47]

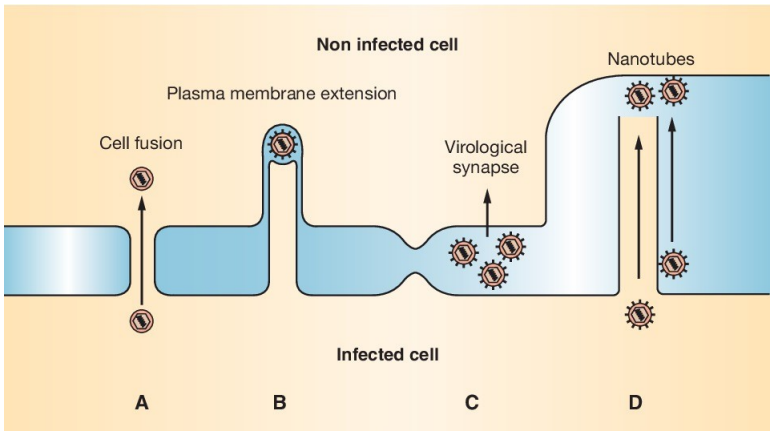

FIGURE 4.10 **Direct cell-to-cell transmission**. Several mechanisms allow infection to be transmitted via cell contacts without the release of free virus particles into the extracellular space. **A**. Due to the presence of viral fusion proteins on the surface of the infected cells and virus receptors on the noninfected cells, fusion of two cells can occur without producing virus particles. This results in the formation of syncytia. This mechanism is often seen with enveloped viruses and with fusion proteins that do not require low pH for fusion. **B**. In the case of poxviruses, extracellular viruses belonging to the so-called extracellular enveloped class of virions remain attached to the surface of the infected cell, where they trigger the formation of a motile, actin-containing, extension of the plasma membrane. This extension is thought to promote contact of the virus with the surface of neighboring cells. **C**. The infected cells can undergo polarization so that progeny viruses are selectively released into a region of the cell periphery intimately in contact with another cell. Such specialized regions are called virological synapses. **D**. Viruses can induce the formation of long actin–containing nanotubes on infected cells. Virions move within nanotubes or on the surface of nanotubes to mediate spread to distant cells. In some cases, nanotubes offer protection from neutralizing antibodies.

Other mechanisms of cell-to-cell transmission require the formation of virus particles, but these are not released freely into the extracellular space. Poxviruses such as vaccinia virus generate infectious particles called extracellular enveloped viruses, most of which not only remain attached to the cell surface after formation but also induce the formation of motile surface extensions through the polymerization of actin inside the cytoplasm.[88,99,252,295] The actin polymerization reaction helps to form membrane extensions that push the virus into contact with the

neighboring cell, thus generating an efficient mechanism for local dissemination in infected tissues.

Structures termed infectious or virological synapses were first described for the transmission of the human T-cell leukemia virus type 1 (HTLV-1).[116] These are areas of intimate contact between infected and uninfected cells reminiscent of immunological synapses. They provide a domain where virus assembly and release are focused with efficient targeting of the virus particles to the next host cell without access of antibodies from the outside.[184] Similar means of transfer have been described for herpesviruses and for the T cell to T-cell transfer of HIV. Dendritic cells that bind HIV-1 via DC-SIGN help to transport the virus to lymph nodes where they present the virus to T cells, providing an efficient system for targeted infection of host cells through virological synapses.[92,137]

Retroviruses as well as herpesviruses and several others also take advantage of nanotubes for cell-to-cell transmission (Fig. 4.10).[120] Nanotubes are long, actin-rich membrane protrusions that, under physiological conditions, mediate communication and the transfer of vesicles and organelles from one cell to another. The use of nanotubes for cell-cell virus spread was first reported for HIV-1.[254] Depending on the cell type, HIV capsids traverse nanotubes within endocytic vesicles, or intact virions surf along the outside of nanotubes, to uninfected cells.[76,124] In macrophages, this provides the advantage of being able to spread in the presence of neutralizing antibodies.[114,298]

Perspectives

The entry and uncoating mechanisms and strategies are highly variable depending on the virus and the target cell. They have in common that the viruses depend critically on the host cell and its activities. To extract the necessary assistance from the cell, viruses make use of the detailed *insider information* they have about the host organisms, their tissues, the cells, and a variety of molecular processes. This information has been acquired during millions of years of coevolution. Thus, although exceptionally simple in structure and composition, viruses are able to elicit exceedingly complex cellular responses. The induction of signals, the activation of endocytic pathways, the exploitation of intracellular targeting systems and molecular motors, and the cell-assisted mechanisms of uncoating provide examples of the ways in which viruses make use of cellular machinery during entry. As details unfold, interesting and important insights about the viruses and their hosts continue to emerge.

The entry program involves the transport of the incoming viruses and capsids through the membranes and the compartments of the cell, the stepwise dismantling of the virus particle, and the release of the genome. Entry and uncoating involve *switches* in particle structure and properties that have to occur in the right place at the right time. It is remarkable how little seems to be left to chance. In the future, these switches need more attention because they provide powerful targets for therapeutic and prophylactic intervention. Advances in cryo-EM and super-resolution microscopy promise to advance our understanding of virus protein architecture and how structure drives function.

Also, the cellular defenses against virus entry, ranging from degradation of incoming viruses to interferon-induced expression of interception proteins in the cytosol, deserve thorough study. After all, it is clear that, of the incoming cell-associated viruses, only a small fraction generally reach the intended goal.

It will be important to focus on the cellular factors involved in infection (ie, on the Trojans and not only on the Trojan horses). They represent new targets for antiviral strategies. How viruses enter tissues and cells in intact organisms remains for the most part territory uncharted. New technologies and model systems are emerging that allow work in complex 3-D tissue culture systems and at the whole animal level. A multidisciplinary approach that combines cell and molecular biology, structural biology, biochemistry, physiology, systems biology, and medicine as central disciplines is required. As important as knowing the pathogens, it will also be important in the spirit of an ancient Greek aphorism to know ourselves (ie, to understand the cell and molecular biology of the host cells).

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REFERENCES

1. Albritton LM, Tseng L, Scadden D, et al. A putative murine ecotropic retrovirus receptor gene codes a multiple membrane spanning protein and confers susceptibility to virus infection. *Cell*. 1989;57:659-666.

2. Alkhatib G, Combadiere C, Broder CC, et al. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science*. 1996;272:1955-1958.

3. Alkhatib G, Liao F, Berger EA, et al. A new SIV co-receptor, STRL33. *Nature*. 1997;388:238.

4. Alvarez CP, Lasala F, Carrillo J, Muñiz O, Corbí AL, Delgado R. C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. J Virol. 2002;76(13):6841-6844. doi:10.1128/jvi.76.13.6841-6844.2002

5. Amara A, Mercer J. Viral apoptotic mimicry. Nat Rev Microbiol. 2015;13(8):461-469. doi:10.1038/nrmicro3469

6. Amstutz B, Gastaldelli M, Kalin S, et al. Subversion of CtBP1-controlled macropinocytosis by human adenovirus serotype 3. *EMBO J*. 2008;27:956-969.

7. Anderson HA, Chen Y, Norkin LC. Bound simian virus 40 translocates to caveolin-enriched membrane domains, and its entry is inhibited by drugs that selectively disrupt caveolae. *Mol Biol Cell*. 1996;7:1825-1834.

8. Aydin I, Weber S, Snijder B, et al. Large scale RNAi reveals the requirement of nuclear envelope breakdown for nuclear import of human papillomaviruses. PLoS Pathog. 2014;10(5):e1004162. doi:10.1371/journal.ppat.1004162

9. Bagchi P, Torres M, Qi L, Tsai B. Selective EMC subunits act as molecular tethers of intracellular organelles exploited during viral entry. Nat Commun. 2020;11(1):1127. doi:10.1038/s41467-020-14967-w

10. Banerjee I, Miyake Y, Nobs SP, et al. Influenza A virus uses the aggresome processing machinery for host cell entry. Science.

2014;346(6208):473-477. doi:10.1126/science.1257037

11. Bartlett AH, Park PW. Proteoglycans in host-pathogen interactions: molecular mechanisms and therapeutic implications. *Expert Rev Mol Med*. 2010;12:e5.

12. Bates P, Young JA, Varmus HE. A receptor for subgroup A Rous sarcoma virus is related to the low density lipoprotein receptor. *Cell*. 1993;74:1043-1051.

13. Battini JL, Rasko JE, Miller AD. A human cell-surface receptor for xenotropic and polytropic murine leukemia viruses: possible role in G protein-coupled signal transduction. *Proc Natl Acad Sci U S A*. 1999;96:1385-1390.

14. Bengali Z, Satheshkumar PS, Moss B. Orthopoxvirus species and strain differences in cell entry. Virology. 2012;433(2):506-512. doi:10.1016/j.virol.2012.08.044

15. Bengali Z, Townsley AC, Moss B. Vaccinia virus strain differences in cell attachment and entry. Virology. 2009;389(1-2):132-140. doi: 10.1016/j.virol.2009.04.012

16. Bergant Marušič M, Ozbun MA, Campos SK, Myers MP, Banks L. Human papillomavirus L2 facilitates viral escape from late endosomes via sorting nexin 17. Traffic. 2012;13(3):455-467. doi:10.1111/j.1600-0854.2011.01320.x

17. Bergelson JM. Virus interactions with mucosal surfaces: alternative receptors, alternative pathways. *Curr Opin Microbiol*. 2003;6:386-391.

18. Bergelson JM, Cunningham JA, Droguett G, et al. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science*. 1997;275:1320-1323.

19. Bergelson JM, Mohanty JG, Crowell RL, et al. Coxsackievirus B3 adapted to growth in RD cells binds to decay-accelerating factor (CD55). *J Virol*. 1995;69:1903-1906.

20. Bergelson JM, Shepley MP, Chan BM, et al. Identification of the integrin VLA-2 as a receptor for echovirus 1. *Science*. 1992;255:1718-1720.

21. Berger EA, Murphy PM, Farber JM. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol*. 1999;17:657-700.

22. Berinstein A, Roivainen M, Hovi T, et al. Antibodies to the vitronectin receptor (integrin alpha V beta 3) inhibit binding and infection of foot-and-mouth disease virus to cultured cells. *J Virol*. 1995;69:2664-2666.

23. Bewley MC, Springer K, Zhang YB, et al. Structural analysis of the mechanism of adenovirus binding to its human cellular receptor, CAR. *Science*. 1999;286:1579-1583.

24. Bhattacharyya S, Zagorska A, Lew ED, et al. Enveloped viruses disable innate immune responses in dendritic cells by direct activation of TAM receptors. Cell Host Microbe. 2013;14(2):136-147. doi:10.1016/j.chom.2013.07.005

25. Boulware DR, Pullen MF, Bangdiwala AS, et al. A randomized trial of hydroxychloroquine as postexposure prophylaxis for Covid-19. N Engl J Med. 2020;383(6):517-525. doi:10.1056/NEJMoa2016638

26. Brabec-Zaruba M, Pfanzagl B, Blaas D, Fuchs R. Site of human rhinovirus RNA uncoating revealed by fluorescent in situ hybridization. *J Virol*. 2009:83:3770-3777.

27. Brandenburg B, Lee LY, Lakadamyali M, et al. Imaging poliovirus entry in live cells. *PLoS Biol*. 2007;5:e183.

28. Brojatsch J, Naughton J, Rolls MM, et al. CAR1, a TNFR-related protein, is a cellular receptor for cytopathic avian leukosis-sarcoma viruses and mediates apoptosis. *Cell*. 1996;87:845-855.

29. Bubeck D, Filman DJ, Cheng N, et al. The structure of the poliovirus 135S cell entry intermediate at 10-angstrom resolution reveals the location of an externalized polypeptide that binds to membranes. *J Virol*. 2005;79:7745-7755.

30. Bubeck D, Filman DJ, Hogle JM. Cryo-electron microscopy reconstruction of a poliovirus-receptor-membrane complex. *Nat Struct Mol Biol*. 2005;12:615-618.

31. Buckley CM, King JS. Drinking problems: mechanisms of macropinosome formation and maturation. FEBS J. 2017;284(22):3778-3790. doi:10.1111/febs.14115

32. Bui M, Whittaker G, Helenius A. The effect of M1 protein and low pH on nuclear import of influenza virus vRNPs. *J Virol*. 1996;70:8391-8401.

33. Bukrinskaya AG, Vorkunova NK, Kornilayeva GV, et al. Influenza virus uncoating in infected cells and effect of rimatidine. *J Gen Virol*. 1982;60:49-59.

34. Byrnes AP, Griffin DE. Large-plaque mutants of Sindbis virus show reduced binding to heparan sulfate, heightened viremia, and slower clearance from the circulation. *J Virol*. 2000;74:644-651.

35. Campanero-Rhodes MA, Smith A, Chai W, et al. N-glycolyl GM1 ganglioside as a receptor for simian virus 40. *J Virol*. 2007;81:12846-12858.

36. Cao W, Henry MD, Borrow P, et al. Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus [see comments]. *Science*. 1998;282:2079-2081.

37. Capon DJ, Ward RH. The CD4-gp120 interaction and AIDS pathogenesis. *Annu Rev Immunol*. 1991;9:649-678.

38. Carette JE, Raaben M, Wong AC, et al. Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. Nature. 2011;477(7364):340-343. doi:10.1038/nature10348

39. Carfi A, Willis SH, Whitbeck JC, et al. Herpes simplex virus glycoprotein D bound to the human receptor HveA. *Mol Cell*. 2001;8:169-179.

40. Carr CM, Kim PS. A spring-loaded mechanism for the conformational change of influenza hemagglutinin. *Cell*. 1993;73:823-832.

41. Chandran B. Early events in Kaposi's sarcoma-associated herpesvirus infection of target cells. *J Virol*. 2010;84:2188-2199.

42. Chandran K, Farsetta DL, Nibert ML. Strategy for nonenveloped virus entry: a hydrophobic conformer of the reovirus membrane penetration protein micro 1 mediates membrane disruption. *J Virol*. 2002;76:9920-9933.

43. Chandran K, Sullivan NJ, Felbor U, et al. Endosomal proteolysis of the Ebola virus glycoprotein is necessary for infection. *Science*. 2005;308:1643-1645.

44. Chen YJ, Liu X, Tsai B. SV40 Hijacks cellular transport, membrane penetration, and disassembly machineries to promote infection. Viruses. 2019;11(10):917. doi:10.3390/v11100917

45. Cherry S. Genomic RNAi screening in Drosophila S2 cells: what have we learned about host-pathogen interactions? *Curr Opin Microbiol*. 2008;11:262-270.

46. Choe H, Farzan M, Sun Y, et al. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell*. 1996;85:1135-1148.

47. Choppin PW, Compans RW. Replication of paramyxoviruses. In: Fraenkel-Conrat H, Wagner RR, eds. Comprehensive Virology. Vol. 4. Plenum; 1975:94-178.

48. Cohen S, Au S, Pante N. How viruses access the nucleus. *Biochim Biophys Acta*. 2010;1813:1634-1645.

49. Collinet C, Stoter M, Bradshaw CR, et al. Systems survey of endocytosis by multiparametric image analysis. *Nature*. 2010;464:243-249.

50. Compans RW, Content J, Duesberg PH. Structure of the ribonucleoprotein of influenza virus. *J Virol*. 1972;10:795-800.

51. Conner SD, Schmid SL. Regulated portals of entry into the cell. *Nature*. 2003;422:37-44.

52. Côté M, Misasi J, Ren T, et al. Small molecule inhibitors reveal Niemann-Pick C1 is essential for Ebola virus infection. Nature. 2011;477(7364):344-348. doi:10.1038/nature10380

53. Cotmore SF, Tattersall P. Parvoviral host range and cell entry mechanisms. *Adv Virus Res*. 2007;70:183-232.

54. Coyne CB, Bergelson JM. Virus-induced Abl and Fyn kinase signals permit coxsackievirus entry through epithelial tight junctions. *Cell*. 2006;124:119-131.

55. Cullen B. Journey to the center of the cell. *Cell*. 2001;105:697-700.

56. Cureton DK, Massol RH, Saffarian S, et al. Vesicular stomatitis virus enters cells through vesicles incompletely coated with clathrin that depend upon actin for internalization. *PLoS Pathog*. 2009;5:e1000394.

57. Daecke J, Fackler OT, Dittmar MT, et al. Involvement of clathrin-mediated endocytosis in human immunodeficiency virus type 1 entry. *J Virol*. 2005;79:1581-1594.

58. Dales S. The uptake and development of vaccinia virus in strain L cells followed with labeled viral deoxyribonucleic acid. *J Cell Biol*. 1963;18:51-72.

59. Dales S. Early events in cell-animal virus interactions. *Bacteriol Rev*. 1973;37:103-135.

60. Damm EM, Pelkmans L, Kartenbeck J, et al. Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. *J Cell Biol*. 2005;168:477-488.

61. Daniels R, Rusan NM, Wadsworth P, Hebert DN. SV40 VP2 and VP3 insertion into ER membranes is controlled by the capsid protein VP1: implications for DNA translocation out of the ER. Mol Cell. 2006;24(6):955-966. doi:10.1016/j.molcel.2006.11.001

62. Day PM, Thompson CD, Schowalter RM, Lowy DR, Schiller JT. Identification of a role for the trans-Golgi network in human papillomavirus 16 pseudovirus infection. J Virol. 2013;87(7):3862-3870. doi:10.1128/JVI.03222-12

63. Delpeut S, Sisson G, Black KM, Richardson CD. Measles virus enters breast and colon cancer cell lines through a PVRL4-mediated macropinocytosis pathway. J Virol. 2017;91(10):e02191-16. doi:10.1128/JVI.02191-16

64. Doherty GJ, McMahon HT. Mechanisms of endocytosis. *Annu Rev Biochem*. 2009;78:857-902. doi:10.1146/annurev.biochem.78.081307.110540

65. Dohner K, Nagel CH, Sodeik B. Viral stop-and-go along microtubules: taking a ride with dynein and kinesins. *Trends Microbiol*. 2005;13:320-327.

66. Donaldson JG. Macropinosome formation, maturation and membrane recycling: lessons from clathrin-independent endosomal membrane systems. Philos Trans R Soc Lond B Biol Sci. 2019;374(1765):20180148. doi:10.1098/rstb.2018.0148

67. Donaldson JG, Porat-Shliom N, Cohen LA. Clathrin-independent endocytosis: a unique platform for cell signaling and PM remodeling. *Cell*

Signal. 2009;21:1-6.

68. Doranz BJ, Rucker J, Yi Y, et al. A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell*. 1996;85:1149-1158.

69. Dorig RE, Marcil A, Chopra A, et al. The human CD46 molecule is a receptor for measles virus (Edmonston strain). *Cell*. 1993;75:295-305.

70. Dupzyk A, Tsai B. Bag2 is a component of a cytosolic extraction machinery that promotes membrane penetration of a nonenveloped virus. J Virol. 2018;92(15):e00607-18. doi:10.1128/JVI.00607-18

71. Dupzyk A, Williams JM, Bagchi P, Inoue T, Tsai B. SGTA-dependent regulation of Hsc70 promotes cytosol entry of simian virus 40 from the endoplasmic reticulum. J Virol. 2017;91(12):e00232-17. doi:10.1128/JVI.00232-17

72. Dveksler GS, Pensiero MN, Cardellichio CB, et al. Cloning of the mouse hepatitis virus (MHV) receptor: expression in human and hamster cell lines confers susceptibility to MHV. *J Virol*. 1991;65:6881-6891.

73. Ehrlich M, Boll W, Van Oijen A, et al. Endocytosis by random initiation and stabilization of clathrin-coated pits. *Cell*. 2004;118:591-605.

74. Engel S, Heger T, Mancini R, et al. The role of endosomes in SV40 entry and infection. *J Virol*. 2011;85:4198-4211.

75. English TJ, Hammer DA. The effect of cellular receptor diffusion on receptor-mediated viral binding using Brownian adhesive dynamics (BRAD) simulations. *Biophys J*. 2005;88:1666-1675.

76. Eugenin EA, Gaskill PJ, Berman JW. Tunneling nanotubes (TNT) are induced by HIV-infection of macrophages: a potential mechanism for intercellular HIV trafficking. Cell Immunol. 2009;254(2):142-148. doi:10.1016/j.cellimm.2008.08.005

77. Ewers H, Römer W, Smith AE, et al. GM1 structure determines SV40-induced membrane invagination and infection. *Nat Cell Biol*. 2010;12:11-18.

78. Ewers H, Smith AE, Sbalzarini IF, et al. Single-particle tracking of murine polyoma virus-like particles on live cells and artificial membranes. *Proc Natl Acad Sci U S A*. 2005;102:15110-15115.

79. Fadok VA, Bratton DL, Frasch SC, et al. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. *Cell Death Differ*. 1998;5:551-562.

80. Farzan M, Choe H, Martin K, et al. Two orphan seven-transmembrane segment receptors which are expressed in CD4-positive cells support simian immunodeficiency virus infection. *J Exp Med*. 1997;186:405-411.

81. Feinberg H, Mitchell DA, Drickamer K, et al. Structural basis for selective recognition of oligosaccharides by DC-SIGN and DC-SIGNR. *Science*. 2001;294:2163-2166.

82. Feire AL, Koss H, Compton T. Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain. *Proc Natl Acad Sci U S A*. 2004;101:15470-15475.

83. Ferner RE, Aronson JK. Chloroquine and hydroxychloroquine in covid-19. *BMJ*. 2020;369:m1432. doi:10.1136/bmj.m1432

84. Fingeroth JD, Weis JJ, Tedder TF, et al. Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. *Proc Natl Acad Sci U S A*. 1984;81:4510-4514.

85. Frade R, Barel M, Ehlin-Henriksson B, et al. gp140, the C3d receptor of human B lymphocytes, is also the Epstein-Barr virus receptor. Proc Natl Acad Sci U S A. 1985;82:1490-1493.

86. Frei AP, Jeon OY, Kilcher S, et al. Direct identification of ligand-receptor interactions on living cells and tissues. Nat Biotechnol. 2012;30(10):997-1001. doi:10.1038/nbt.2354

87. Fricks CE, Hogle JM. Cell-induced conformational change in poliovirus: externalization of the amino terminus of VP1 is responsible for liposome binding. *J Virol*. 1990;64: 1934-1945.

88. Frischknecht F, Way M. Surfing pathogens and the lessons learned for actin polymerization. *Trends Cell Biol*. 2001;11:30-38.

89. Fuchs R, Blaas D. Uncoating of human rhinoviruses. *Rev Med Virol*. 2010;20:281-297.

90. Gavrilovskaya IN, Shepley M, Shaw R, et al. beta3 Integrins mediate the cellular entry of hantaviruses that cause respiratory failure. Proc Natl Acad Sci U S A. 1998;95:7074-7079.

91. Geiger R, Andritschke D, Friebe S, et al. BAP31 and BiP are essential for dislocation of SV40 from the endoplasmic reticulum to the cytosol. Nat Cell Biol. 2011;13(11):1305-1314. doi:10.1038/ncb2339

92. Geijtenbeek TB, Kwon DS, Torensma R, et al. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell*. 2000;100:587-597.

93. Geleris J, Sun Y, Platt J, et al. Observational study of hydroxychloroquine in hospitalized patients with Covid-19. N Engl J Med. 2020;382(25):2411-2418. doi:10.1056/NEJMoa2012410

94. Geraghty RJ, Krummenacher C, Cohen GH, et al. Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science*. 1998;280:1618-1620.

95. Gilbert J, Benjamin T. Uptake pathway of polyomavirus via ganglioside GD1a. J Virol. 2004;78(22):12259-12267.

doi:10.1128/JVI.78.22.12259-12267.2004

96. Goodwin EC, Lipovsky A, Inoue T, et al. BiP and multiple DNAJ molecular chaperones in the endoplasmic reticulum are required for efficient simian virus 40 infection. mBio. 2011;2(3):e00101-11. doi: 10.1128/mBio.00101-11

97. Greber U. Signalling in viral entry. *Cell Mol Life Sci*. 2002;59:608-626.

98. Greber UF, Fassati A. Nuclear import of viral DNA genomes. *Traffic*. 2003;4:136-143.

99. Greber UF, Way M. A superhighway to virus infection. *Cell*. 2006;124:741-754.

100. Greve JM, Davis G, Meyer AM, et al. The major human rhinovirus receptor is ICAM-1. *Cell*. 1989;56:839-847.

101. Gruenberg J. Viruses and endosome membrane dynamics. *Curr Opin Cell Biol*. 2009;21:582-588.

102. Gruenberg J, Stenmark H. The biogenesis of multivesicular endosomes. *Nat Rev Mol Cell Biol*. 2004;5:317-323.

103. Guedán A, Donaldson CD, Caroe ER, Cosnefroy O, Taylor IA, Bishop KN. HIV-1 requires capsid remodelling at the nuclear pore for nuclear entry and integration. PLoS Pathog. 2021;17(9):e1009484. doi:10.1371/journal.ppat.1009484

104. Harbison CE, Chiorini JA, Parrish CR. The parvovirus capsid odyssey: from the cell surface to the nucleus. *Trends Microbiol*. 2008;16:208-214.

105. Heinz FX, Allison SL. The machinery for flavivirus fusion with host cell membranes. *Curr Opin Microbiol*. 2001;4:450-455.

106. Helenius A, Kartenbeck J, Simons K, et al. On the entry of Semliki forest virus into BHK-21 cells. *J Cell Biol*. 1980;84:404-420.

107. Helmuth JA, Burckhardt CJ, Greber UF, et al. Shape reconstruction of subcellular structures from live cell fluorescence microscopy images. *J Struct Biol*. 2009;167:1-10.

108. Herrmann T, Torres R, Salgado EN, et al. Functional refolding of the penetration protein on a non-enveloped virus. Nature. 2021;590(7847):666-670. doi:10.1038/s41586-020-03124-4

109. Hewish MJ, Takada Y, Coulson BS. Integrins alpha2beta1 and alpha4beta1 can mediate SA11 rotavirus attachment and entry into cells. *J Virol*. 2000;74:228-236.

110. Hirsch AJ. The use of RNAi-based screens to identify host proteins involved in viral replication. *Future Microbiol*. 2010;5:303-311.

111. Hofer F, Gruenberger M, Kowalski H, et al. Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. *Proc Natl Acad Sci U S A*. 1994;91:1839-1842.

112. Hoffmann M, Kleine-Weber H, Schroeder S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell. 2020;181(2):271-280.e8. doi: 10.1016/j.cell.2020.02.052

113. Hoffmann M, Mösbauer K, Hofmann-Winkler H, et al. Chloroquine does not inhibit infection of human lung cells with SARS-CoV-2. Nature. 2020;585(7826):588-590. doi:10.1038/s41586-020-2575-3

114. Hogle JM. Poliovirus cell entry: common structural themes in viral cell entry pathways. *Annu Rev Microbiol*. 2002;56:677-702.

115. Huotari J, Helenius A. Endosome maturation. *EMBO J*. 2011;30:3481-3500.

116. Igakura T, Stinchcombe JC, Goon PK, et al. Spread of HTLV-I between lymphocytes by virus-induced polarization of the cytoskeleton. *Science*. 2003;299:1713-1716.

117. Jackson T, Blakemore W, Newman JW, et al. Foot-and-mouth disease virus is a ligand for the high-affinity binding conformation of integrin alpha5beta1: influence of the leucine residue within the RGDL motif on selectivity of integrin binding. *J Gen Virol*. 2000;81:1383-1391.

118. Jackson CB, Farzan M, Chen B, Choe H. Mechanisms of SARS-CoV-2 entry into cells. Nat Rev Mol Cell Biol. 2022;23(1):3-20. doi:10.1038/s41580-021-00418-x

119. Jae LT, Raaben M, Herbert AS, et al. Virus entry. Lassa virus entry requires a trigger-induced receptor switch. Science. 2014;344(6191):1506-10. doi:10.1126/science.1252480

120. Jansens RJJ, Tishchenko A, Favoreel HW. Bridging the gap: virus long-distance spread via tunneling nanotubes. J Virol. 2020;94(8). doi: 10.1128/JVI.02120-19

121. Johannsdottir HK, Mancini R, Kartenbeck J, et al. Host cell factors and functions involved in vesicular stomatitis virus entry. *J Virol*. 2009;83:440-453.

122. Joklik WK. The intracellular uncoating of poxvirus DNA. I. The fate of radioactively-labeled rabbitpox virus. J Mol Biol. 1964;8:263-276. doi:10.1016/s0022-2836(64)80136-4

123. Joklik WK. The intracellular uncoating of poxvirus DNA. II. The molecular basis of the uncoating process. J Mol Biol. 1964;8:277-288. doi:10.1016/s0022-2836(64)80137-6

124. Kadiu I, Gendelman HE. Human immunodeficiency virus type 1 endocytic trafficking through macrophage bridging conduits facilitates spread of infection. J Neuroimmune Pharmacol. 2011;6(4):658-275. doi:10.1007/s11481-011-9298-z

125. Kann M, Sodeik B, Vlachou A, et al. Phosphorylation-dependent binding of hepatitis B virus core particles to the nuclear pore complex. *J Cell Biol*. 1999;145:45-55.

126. Karjalainen M, Kakkonen E, Upla P, et al. A Raft-derived, Pak1-regulated entry participates in alpha2beta1 integrin-dependent sorting to caveosomes. *Mol Biol Cell*. 2008;19:2857-2869.

127. Kates J, Beeson J. Ribonucleic acid synthesis in vaccnina virus. I. The mechanism of synthesis and release of RNA in vaccinia cores. J Mol Biol. 1970;50:1-18.

128. Kerr MC, Lindsay MR, Luetterforst R, et al. Visualisation of macropinosome maturation by the recruitment of sorting nexins. J Cell Sci. 2006;119(Pt 19):3967-3980. doi:10.1242/jcs.03167

129. Kienberger F, Zhu R, Moser R, et al. Monitoring RNA release from human rhinovirus by dynamic force microscopy. *J Virol*. 2004;78:3203-3209.

130. Kilcher S, Mercer J. DNA virus uncoating. Virology. 2015;479-480:578-590. doi: 10.1016/j.virol.2015.01.024

131. Kilcher S, Schmidt FI, Schneider C, Kopf M, Helenius A, Mercer J. siRNA screen of early poxvirus genes identifies the AAA+ ATPase D5 as the virus genome-uncoating factor. Cell Host Microbe. 2014;15(1):103-112. doi:10.1016/j.chom.2013.12.008

132. Klenk HD, Rott R, Orlich M, et al. Activation of influenza A viruses by trypsin treatment. *Virology*. 1975;68:426-439.

133. Klimstra WB, Nangle EM, Smith MS, et al. DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. *J Virol*. 2003;77:12022-12032.

134. Kolokoltsov AA, Deniger D, Fleming EH, et al. Small interfering RNA profiling reveals key role of clathrin-mediated endocytosis and early endosome formation for infection by respiratory syncytial virus. *J Virol*. 2007;81:7786-7800.

135. Kondratowicz AS, Lennemann NJ, Sinn PL, et al. T-cell immunoglobulin and mucin domain 1 (TIM-1) is a receptor for Zaire Ebolavirus and Lake Victoria Marburgvirus. Proc Natl Acad Sci U S A. 2011;108(20):8426-8431. doi:10.1073/pnas.1019030108

136. Kukura P, Ewers H, Müller C, Renn A, Helenius A, Sandoghdar V. High-speed nanoscopic tracking of the position and orientation of a single virus. Nat Methods. 2009;6(12):923-927. doi:10.1038/nmeth.1395

137. Kwon DS, Gregorio G, Bitton N, et al. DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection. *Immunity*. 2002;16:135-144.

138. Lajoie P, Nabi IR. Regulation of raft-dependent endocytosis. *J Cell Mol Med*. 2007;11:644-653.

139. Lakadamyali M, Rust MJ, Babcock HP, et al. Visualizing infection of individual influenza viruses. *Proc Natl Acad Sci U S A*. 2003;100:9280-9285.

140. Lehmann MJ, Sherer NM, Marks CB, et al. Actin- and myosin-driven movement of viruses along filopodia precedes their entry into cells. *J Cell Biol*. 2005;170:317-325.

141. Lewis PF, Emerman M. Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J Virol*. 1994;68:510-516.

142. Li Q, Fischer E, Cohen JI. Cell surface THY-1 contributes to human cytomegalovirus entry via a macropinocytosis-like process. J Virol. 2016;90(21):9766-9781. doi:10.1128/JVI.01092-16

143. Li S, Sun Z, Pryce R, et al. Acidic pH-induced conformations and LAMP1 binding of the Lassa virus glycoprotein spike. PLoS Pathog. 2016;12(2):e1005418. doi:10.1371/journal.ppat.1005418

144. Li W, Moore MJ, Vasilieva N, et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature. 2003;426(6965):450-454. doi:10.1038/nature02145

145. Liberali P, Ramo P, Pelkmans L. Protein kinases: starting a molecular systems view of endocytosis. *Annu Rev Cell Dev Biol*. 2008;24:501-523.

146. Lipovsky A, Popa A, Pimienta G, et al. Genome-wide siRNA screen identifies the retromer as a cellular entry factor for human papillomavirus. Proc Natl Acad Sci U S A. 2013;110(18):7452-7457. doi:10.1073/pnas.1302164110

147. Locker JK, Griffiths G. An unconventional role for cytoplasmic disulfide bonds in vaccinia virus proteins. J Cell Biol. 1999;144(2):267-279.

148. Loetscher M, Amara A, Oberlin E, et al. TYMSTR, a putative chemokine receptor selectively expressed in activated T cells, exhibits HIV-1 coreceptor function. *Curr Biol*. 1997;7:652-660.

149. Lonberg-Holm K, Philipson L. Early interaction between animal viruses and cells. *Monogr Virol*. 1974;9:1-149.

150. Lozach PY, Huotari J, Helenius A. Late-penetrating viruses. *Curr Opin Virol*. 2011;1:35-43.

151. Lozach PY, Kuhbacher A, Meier R, et al. DC-SIGN as a receptor for phleboviruses. *Cell Host Microbe*. 2011;10:75-88.

152. Lozach PY, Lortat-Jacob H, de Lacroix de Lavalette A, et al. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. *J Biol Chem*. 2003;278:20358-20366.

153. Lusso P, Secchiero P, Crowley RW, et al. CD4 is a critical component of the receptor for human herpesvirus 7: interference with human

immunodeficiency virus. *Proc Natl Acad Sci U S A*. 1994;91:3872-3876.

154. Lyman MG, Enquist LW. Herpesvirus interactions with the host cytoskeleton. *J Virol*. 2009;83:2058-2066.

155. Ma H, Kim AS, Kafai NM, et al. LDLRAD3 is a receptor for Venezuelan equine encephalitis virus. Nature. 2020;588(7837):308-314. doi:10.1038/s41586-020-2915-3

156. Maddon P, Dalgleish A, McDougal J, et al. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell*. 1986;47:333-348.

157. Marechal V, Prevost MC, Petit C, et al. Human immunodeficiency virus type 1 entry into macrophages mediated by macropinocytosis. *J Virol*. 2001;75:11166-11177.

158. Marsh M, Bron R. SFV infection in CHO cells: cell-type specific restrictions to productive virus entry at the cell surface. J Cell Sci. 1997;110(Pt 1):95-103.

159. Marsh M, Helenius A. Virus entry into animal cells. *Adv Virus Res*. 1989;36:107-151.

160. Marsh M, Helenius A. Virus entry: open sesame. *Cell*. 2006;124:729-740.

161. Martin K, Helenius A. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. *Cell*. 1991;67:117-130.

162. Matlin KS, Reggio H, Helenius A, et al. Infectious entry pathway of influenza virus in a canine kidney cell line. *J Cell Biol*. 1982;91:601-613.

163. Matlin KS, Reggio H, Helenius A, et al. The pathway of vesicular stomatitis virus entry leading to infection. *J Mol Biol*. 1982;156:609-631.

164. Maul GG, Rovera G, Vorbrodt A, et al. Membrane fusion as a mechanism of Simian virus 40 entry into different cellular compartments. *J Virol*. 1978;28:936-944.

165. Mauri DN, Ebner R, Montgomery RI, et al. LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. *Immunity*. 1998;8:21-30.

166. Mayor S, Pagano RE. Pathways of clathrin-independent endocytosis. *Nat Rev Mol Cell Biol*. 2007;8:603-612.

167. Medina-Kauwe LK. Endocytosis of adenovirus and adenovirus capsid proteins. *Adv Drug Deliv Rev*. 2003;55:1485-1496.

168. Meertens L, Carnec X, Lecoin MP, et al. The TIM and TAM families of phosphatidylserine receptors mediate dengue virus entry. Cell Host Microbe. 2012;12(4):544-557. doi:10.1016/j.chom.2012.08.009

169. Meier O, Boucke K, Hammer SV, et al. Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. *J Cell Biol*. 2002;158:1119-1131.

170. Meier O, Greber UF. Adenovirus endocytosis. *J Gene Med*. 2003;5:451-462.

171. Mellman I. Endocytosis and molecular sorting. *Annu Rev Cell Dev Biol*. 1996;12:575-625.

172. Mendelsohn CL, Wimmer E, Racaniello VR. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. *Cell*. 1989;56:855-865.

173. Mercer J, Greber UF. Virus interactions with endocytic pathways in macrophages and dendritic cells. Trends Microbiol. 2013;21(8):380-388. doi:10.1016/j.tim.2013.06.001

174. Mercer J, Helenius A. Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. *Science*. 2008;320:531-535.

175. Mercer J, Helenius A. Virus entry by macropinocytosis. *Nat Cell Biol*. 2009;11:510-520.

176. Mercer J, Helenius A. Gulping rather than sipping: macropinocytosis as a way of virus entry. Curr Opin Microbiol. 2012;15(4):490-499. doi:10.1016/j.mib.2012.05.016

177. Mercer J, Schelhaas M, Helenius A. Virus entry by endocytosis. *Annu Rev Biochem*. 2010;79:803-833.

178. Mercer J, Snijder B, Sacher R, et al. RNAi screening reveals proteasome- and Cullin3-dependent stages in vaccinia virus infection. Cell Rep. 2012;2(4):1036-1047. doi:10.1016/j.celrep.2012.09.003

179. Miller D, Miller A. A family of retroviruses that utilize related phosphate transporters for cell entry. *J Virol*. 1994;68:8270-8276.

180. Miller M, Farnet C, Bushman F. Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. *J Virol*. 1997;71:5382-5390.

181. Mondor I, Ugolini S, Sattentau QJ. Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and requires cell surface heparans. *J Virol*. 1998;72:3623-3634.

182. Morosky S, Wells AI, Lemon K, et al. The neonatal Fc receptor is a pan-echovirus receptor. Proc Natl Acad Sci U S A. 2019;116(9):3758-3763. doi:10.1073/pnas.1817341116

183. Mothes W, Boerger AL, Narayan S, et al. Retroviral entry mediated by receptor priming and low pH triggering of an envelope glycoprotein.

Cell. 2000;103:679-689.

184. Mothes W, Sherer NM, Jin J, et al. Virus cell-to-cell transmission. *J Virol*. 2010;84:8360-8368.

185. Moyer CL, Wiethoff CM, Maier O, et al. Functional genetic and biophysical analyses of membrane disruption by human adenovirus. *J Virol*. 2011;85:2631-2641.

186. Nemerow GR. Cell receptors involved in adenovirus entry. *Virology*. 2000;274:1-4.

187. Neu U, Stehle T, Atwood WJ. The Polyomaviridae: contributions of virus structure to our understanding of virus receptors and infectious entry. *Virology*. 2009;384:389-399.

188. Nicola AV, Hou J, Major EO, et al. Herpes simplex virus type 1 enters human epidermal keratinocytes, but not neurons, via a pH-dependent endocytic pathway. *J Virol*. 2005;79:7609-7616.

189. Nisole S, Saib A. Early steps of retrovirus replicative cycle. *Retrovirology*. 2004;1:9.

190. Nonnenmacher M, Weber T. Adeno-associated virus 2 infection requires endocytosis through the CLIC/GEEC pathway. Cell Host Microbe. 2011;10(6):563-576. doi:10.1016/j.chom.2011.10.014

191. Norkin LC. Caveolae in the uptake and targeting of infectious agents and secreted toxins. *Adv Drug Deliv Rev*. 2001;49:301-315.

192. Norkin LC, Anderson HA, Wolfrom SA, Oppenheim A. Caveolar endocytosis of simian virus 40 is followed by brefeldin A-sensitive transport to the endoplasmic reticulum, where the virus disassembles. J Virol. 2002;76(10):5156-5166. doi:10.1128/jvi.76.10.5156-5166.2002

193. Novoa RR, Calderita G, Arranz R, et al. Virus factories: associations of cell organelles for viral replication and morphogenesis. *Biol Cell*. 2005;97:147-172.

194. O'Hara B, Johann S, Klinger H, et al. Characterization of a human gene conferring sensitivity to infection by gibbon ape leukemia virus. *Cell Growth Differ*. 1990;1:119-127.

195. Ojala PM, Sodeik B, Ebersold MW, et al. Herpes simplex virus type 1 entry into host cells: reconstitution of capsid binding and uncoating at the nuclear pore complex in vitro. *Mol Cell Biol*. 2000;20:4922-4931.

196. O'Neill RE, Jaskunas R, Blobel G, et al. Nuclear import of influenza virus RNA can be mediated by viral nucleoprotein and transport factors required for protein import. *J Biol Chem*. 1995;270:22701-22704.

197. Ou T, Mou H, Zhang L, Ojha A, Choe H, Farzan M. Hydroxychloroquine-mediated inhibition of SARS-CoV-2 entry is attenuated by TMPRSS2. PLoS Pathog. 2021;17(1):e1009212. doi:10.1371/journal.ppat.1009212

198. Pante N, Kann M. Nuclear pore complex is able to transport macromolecules with diameters of about 39 nm. *Mol Biol Cell*. 2002;13:425-434.

199. Parton RG, Simons K. The multiple faces of caveolae. *Nat Rev Mol Cell Biol*. 2007;8:185-194.

200. Pasdeloup D, Blondel D, Isidro AL, et al. Herpesvirus capsid association with the nuclear pore complex and viral DNA release involve the nucleoporin CAN/Nup214 and the capsid protein pUL25. *J Virol*. 2009;83:6610-6623.

201. Peiris JSM, Porterfileld JS. Antibody mediated enhancement of flavivirus replication in macrophage-like cell lines. *Nature*. 1979;282:509-511.

202. Pelkmans L, Fava E, Grabner H, et al. Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. *Nature*. 2005;436:78-86.

203. Pelkmans L, Puntener D, Helenius A. Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. *Science*. 2002;296:535-539.

204. Pelkmans L, Zerial M. Kinase-regulated quantal assemblies and kiss-and-run recycling of caveolae. *Nature*. 2005;436:128-133.

205. Pileri P, Uematsu Y, Campagnoli S, et al. Binding of hepatitis C virus to CD81. *Science*. 1998;282:938-941.

206. Pillay S, Meyer NL, Puschnik AS, et al. An essential receptor for adeno-associated virus infection. Nature. 2016;530(7588):108-112. doi:10.1038/nature16465

207. Pim D, Broniarczyk J, Bergant M, Playford MP, Banks L. A novel PDZ domain interaction mediates the binding between human papillomavirus 16 L2 and sorting Nexin 27 and modulates virion trafficking. J Virol. 2015;89(20):10145-10155. doi:10.1128/JVI.01499-15

208. Pinto LH, Holsinger LJ, Lamb RA. Influenza virus M2 protein has ion channel activity. *Cell*. 1992;69:1-20.

209. Pohlmann S, Baribaud F, Doms RW. DC-SIGN and DC-SIGNR: helping hands for HIV. *Trends Immunol*. 2001;22:643-646.

210. Popa A, Zhang W, Harrison MS, et al. Direct binding of retromer to human papillomavirus type 16 minor capsid protein L2 mediates endosome exit during viral infection. PLoS Pathog. 2015;11(2):e1004699. doi:10.1371/journal.ppat.1004699

211. Poranen MM, Daugelavicius R, Bamford DH. Common principles in viral entry. *Annu Rev Microbiol*. 2002;56:521-538.

212. Pyeon D, Pearce SM, Lank SM, et al. Establishment of human papillomavirus infection requires cell cycle progression. *PLoS Pathog*. 2009;5:e1000318.

213. Qian M, Cai D, Verhey KJ, et al. A lipid receptor sorts polyomavirus from the endolysosome to the endoplasmic reticulum to cause infection. *PLoS Pathog*. 2009;5:e1000465.

214. Rabe B, Vlachou A, Pante N, et al. Nuclear import of hepatitis B virus capsids and release of the viral genome. *Proc Natl Acad Sci U S A*. 2003;100:9849-9854.

215. Radtke K, Kieneke D, Wolfstein A, et al. Plus- and minus-end directed microtubule motors bind simultaneously to herpes simplex virus capsids using different inner tegument structures. *PLoS Pathog*. 2010;6:e1000991.

216. Ramage H, Cherry S. Virus-host interactions: from unbiased genetic screens to function. Annu Rev Virol. 2015;2(1):497-524. doi:10.1146/annurev-virology-100114-055238

217. Rand RP. Interacting phospholipid bilayers: measured forces and induced structural changes. *Annu Rev Biophys Bioeng*. 1981;10:277-314.

218. Ravindran MS, Bagchi P, Inoue T, Tsai B. A non-enveloped virus hijacks host disaggregation machinery to translocate across the endoplasmic reticulum membrane. PLoS Pathog. 2015;11(8):e1005086. doi:10.1371/journal.ppat.1005086

219. Razani B, Woodman SE, Lisanti MP. Caveolae: from cell biology to animal physiology. *Pharmacol Rev*. 2002;54:431-467.

220. Rees PJ, Dimmock NJ. Electrophoretic separation of influenza virus ribonucleoproteins. *J Gen Virol*. 1981;53:125-132.

221. Richterova Z, Liebl D, Horak M, et al. Caveolae are involved in the trafficking of mouse polyomavirus virions and artificial VP1 pseudocapsids toward cell nuclei. *J Virol*. 2001;75:10880-10891.

222. Rizopoulos Z, Balistreri G, Kilcher S, et al. Vaccinia virus infection requires maturation of macropinosomes. Traffic. 2015;16(8):814-831. doi:10.1111/tra.12290

223. Roivainen M, Piirainen L, Hovi T, et al. Entry of coxsackievirus A9 into host cells: specific interactions with alpha v beta 3 integrin, the vitronectin receptor. *Virology*. 1994;203:357-365.

224. Römer W, Berland L, Chambon V, et al. Shiga toxin induces tubular membrane invaginations for its uptake into cells. *Nature*. 2007;450:670-675.

225. Rossmann MG, He Y, Kuhn RJ. Picornavirus-receptor interactions. *Trends Microbiol*. 2002;10:324-331.

226. Roth SL, Whittaker GR. Promotion of vesicular stomatitis virus fusion by the endosome-specific phospholipid bis(monoacylglycero)phosphate (BMP). *FEBS Lett*. 2011;585:865-869.

227. Rucker J, Edinger AL, Sharron M, et al. Utilization of chemokine receptors, orphan receptors, and herpesvirus-encoded receptors by diverse human and simian immunodeficiency viruses. *J Virol*. 1997;71:8999-9007.

228. Rust MJ, Lakadamyali M, Zhang F, et al. Assembly of endocytic machinery around individual influenza viruses during viral entry. *Nat Struct Mol Biol*. 2004;11:567-573.

229. Saeed MF, Kolokoltsov AA, Albrecht T, et al. Cellular entry of Ebola virus involves uptake by a macropinocytosis-like mechanism and subsequent trafficking through early and late endosomes. *PLoS Pathog*. 2010;6:e1001110.

230. Saftig P, Klumperman J. Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. *Nat Rev Mol Cell Biol*. 2009;10:623-635.

231. Sattentau Q. Avoiding the void: cell-to-cell spread of human viruses. *Nat Rev Microbiol*. 2008;6:815-826.

232. Savarino A, Boelaert JR, Cassone A, Majori G, Cauda R. Effects of chloroquine on viral infections: an old drug against today's diseases? Lancet Infect Dis. 2003;3(11):722-727. doi:10.1016/s1473-3099(03)00806-5

233. Schelhaas M, Ewers H, Rajamaki ML, et al. Human papillomavirus type 16 entry: retrograde cell surface transport along actin-rich protrusions. *PLoS Pathog*. 2008;4: e1000148.

234. Schelhaas M, Malmstrom J, Pelkmans L, et al. Simian virus 40 depends on ER protein folding and quality control factors for entry into host cells. *Cell*. 2007;131(3):516-529. doi:10.1016/j.cell.2007.09.038.

235. Schelhaas M, Shah B, Holzer M, et al. Entry of human papillomavirus type 16 by actin-dependent, clathrin- and lipid raft-independent endocytosis. PLoS Pathog. 2012;8(4):e1002657. doi:10.1371/journal.ppat.1002657

236. Schmidt FI, Bleck CK, Reh L, et al. Vaccinia virus entry is followed by core activation and proteasome-mediated release of the immunomodulatory effector VH1 from lateral bodies. Cell Rep. 2013;4(3):464-476. doi:10.1016/j.celrep.2013.06.028

237. Scott CC, Gruenberg J. Ion flux and the function of endosomes and lysosomes: pH is just the start: the flux of ions across endosomal membranes influences endosome function not only through regulation of the luminal pH. *Bioessays*. 2011;33:103-110.

238. Seaman MNJ. The retromer complex: from genesis to revelations. Trends Biochem Sci. 2021;46(7):608-620. doi:10.1016/j.tibs.2020.12.009

239. Seisenberger G, Ried MU, Endress T, et al. Real-time single-molecule imaging of the infection pathway of an adeno-associated virus. *Science*. 2001;294:1929-1932.

240. Seth P, FitzGerald DJP, Willigham MC, et al. Role of a low-pH environment in adenovirus enhancement of the toxicity of a *Pseudomonas*

exotoxin-epidermal growth factor conjugate. *J Virol*. 1984;51:650-655.

241. Shafren DR, Bates RC, Agrez MV, et al. Coxsackieviruses B1, B3, and B5 use decay accelerating factor as a receptor for cell attachment. *J Virol*. 1995;69:3873-3877.

242. Shimojima M, Ikeda Y, Kawaoka Y. The mechanism of Axl-mediated Ebola virus infection. J Infect Dis. 2007;196(Suppl 2):S259-S263. doi:10.1086/520594

243. Sieczkarski SB, Brown HA, Whittaker GR. Role of protein kinase C beta II in influenza virus entry via late endosomes. *J Virol*. 2003;77:460-469.

244. Sieczkarski SB, Whittaker GR. Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. *J Virol*. 2002;76:10455-10464.

245. Sieczkarski SB, Whittaker GR. Differential requirements of Rab5 and Rab7 for endocytosis of influenza and other enveloped viruses. *Traffic*. 2003;4:333-343.

246. Silverstein SC, Astell C, Levin DH, et al. The mechanisms of reovirus uncoating and gene activation in vivo. *Virology*. 1972;47:797-806.

247. Simmons G, Reeves JD, Grogan CC, et al. DC-SIGN and DC-SIGNR bind ebola glycoproteins and enhance infection of macrophages and endothelial cells. *Virology*. 2003;305:115-123.

248. Singh I, Helenius A. Role of ribosomes in Semliki Forest virus nucleocapsid uncoating. *J Virol*. 1992;66:7049-7058.

249. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem*. 2000;69:531-569.

250. Smit JM, Waarts BL, Kimata K, et al. Adaptation of alphaviruses to heparan sulfate: interaction of Sindbis and Semliki Forest viruses with liposomes containing lipid-conjugated heparin. *J Virol*. 2002;76:10128-10137.

251. Smith GA, Enquist LW. Break ins and break outs: viral interactions with the cytoskeleton of mammalian cells. *Annu Rev Cell Dev Biol*. 2002;18:135-161.

252. Smith GL, Murphy BJ, Law M. Vaccinia virus motility. *Annu Rev Microbiol*. 2003;57: 323-342.

253. Sodeik B. Mechanisms of viral transport in the cytoplasm. *Trends Microbiol*. 2000;8: 465-472.

254. Sowinski S, Jolly C, Berninghausen O, et al. Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission. Nat Cell Biol. 2008;10(2):211-219. doi:10.1038/ncb1682

255. Spear PG, Eisenberg RJ, Cohen GH. Three classes of cell surface receptors for alphaherpesvirus entry. *Virology*. 2000;275:1-8.

256. Stadler K, Allison SL, Schalich J, et al. Proteolytic activation of tick-borne encephalitis virus by furin. *J Virol*. 1997;71:8475-8481.

257. Stang E, Kartenbeck J, Parton RG. Major histocompatibility complex class I molecules mediate association of SV40 with caveolae. *Mol Biol Cell*. 1997;8:47-57.

258. Staunton DE, Merluzzi VJ, Rothlein R, et al. A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. *Cell*. 1989;56:849-853.

259. Stewart PL, Dermody TS, Nemerow GR. Structural basis of nonenveloped virus cell entry. *Adv Protein Chem*. 2003;64:455-491.

260. Stewart PL, Nemerow GR. Cell integrins: commonly used receptors for diverse viral pathogens. *Trends Microbiol*. 2007;15:500-507.

261. Strunze S, Engelke MF, Wang IH, et al. Kinesin-1-mediated capsid disassembly and disruption of the nuclear pore complex promote virus infection. Cell Host Microbe. 2011;10(3):210-223. doi:10.1016/j.chom.2011.08.010

262. Strunze S, Trotman LC, Boucke K, Greber UF. Nuclear targeting of adenovirus type 2 requires CRM1-mediated nuclear export. Mol Biol Cell. 2005;16(6):2999-3009. doi:10.1091/mbc.e05-02-0121

263. Sugrue RJ, Hay AJ. Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel. *Virology*. 1991;180:617-624.

264. Suikkanen S, Saajarvi K, Hirsimaki J, et al. Role of recycling endosomes and lysosomes in dynein-dependent entry of canine parvovirus. *J Virol*. 2002;76:4401-4411.

265. Sun P, Yamamoto H, Suetsugu S, Miki H, Takenawa T, Endo T. Small GTPase Rah/Rab34 is associated with membrane ruffles and macropinosomes and promotes macropinosome formation. J Biol Chem. 2003;278(6):4063-4071. doi:10.1074/jbc.M208699200

266. Suomalainen M, Nakano MY, Keller S, et al. Microtubule-dependent plus- and minus end-directed motilities are competing processes for nuclear targeting of adenovirus. *J Cell Biol*. 1999;144:657-672.

267. Swanson JA, Watts C. Macropinocytosis. *Trends Cell Biol*. 1995;5:424-428.

268. Szklarczyk OM, González-Segredo N, Kukura P, et al. Receptor concentration and diffusivity control multivalent binding of Sv40 to membrane bilayers. PLoS Comput Biol. 2013;9(11):e1003310. doi:10.1371/journal.pcbi.1003310

269. Tailor CS, Nouri A, Lee CG, et al. Cloning and characterization of a cell surface receptor for xenotropic and polytropic murine leukemia

viruses. *Proc Natl Acad Sci U S A*. 1999;96:927-932.

270. Takeuchi Y, Vile RG, Simpson G, et al. Feline leukemia virus subgroup B uses the same cell surface receptor as gibbon ape leukemia virus. *J Virol*. 1992;66:1219-1222.

271. Tassaneetrithep B, Burgess TH, Granelli-Piperno A, et al. DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med*. 2003;197:823-829.

272. Tomko RP, Xu R, Philipson L. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc Natl Acad Sci U S A*. 1997;94:3352-3356.

273. Tosteson MT, Chow M. Characterization of the ion channels formed by poliovirus in planar lipid membranes. *J Virol*. 1997;71:507-511.

274. Trotman L, Mosberger N, Fornerod M, et al. Import of adenovirus DNA involves the nuclear pore complex receptor CAN/Nup214 and histone H1. *Nat Cell Biol*. 2001;3:1092-1100.

275. Tsai B, Gilbert JM, Stehle T, Lencer W, Benjamin TL, Rapoport TA. Gangliosides are receptors for murine polyoma virus and SV40. EMBO J. 2003;22(17):4346-4355. doi:10.1093/emboj/cdg439

276. Tsai B, Inoue T. A virus takes an “L” turn to find its receptor. *Cell Host Microbe*. 2010;8:301-302.

277. Valiya Veettil M, Sadagopan S, Kerur N, et al. Interaction of c-Cbl with myosin IIA regulates bleb associated macropinocytosis of Kaposi’s sarcoma-associated herpesvirus. *PLoS Pathog*. 2010;6:e1001238.

278. van Deurs B, Petersen OW, Olsnes S, et al. The ways of endocytosis. *Int Rev Cytol*. 1989;117:131-177.

279. van Deurs B, Roepstorff K, Hommelgaard AM, et al. Caveolae: anchored, multifunctional platforms in the lipid ocean. *Trends Cell Biol*. 2003;13:92-100.

280. van Zeijl M, Johann S, Closs E, et al. A human amphotropic retrovirus receptor is a second member of the gibbon ape leukemia virus receptor family. *Proc Natl Acad Sci U S A*. 1994;91:1168-1172.

281. Vihinen-Ranta M, Suikkanen S, Parrish CR. Pathways of cell infection by parvoviruses and adeno-associated viruses. *J Virol*. 2004;78:6709-6714.

282. Vincent MJ, Bergeron E, Benjannet S, et al. Chloroquine is a potent inhibitor of SARS coronavirus infection and spread. *Virol J*. 2005;2:69. doi:10.1186/1743-422X-2-69

283. Vonderheit A, Helenius A. Rab7 associates with early endosomes to mediate sorting and transport of Semliki Forest virus to late endosomes. *PLoS Biol*. 2005;3:e233.

284. Walczak CP, Ravindran MS, Inoue T, Tsai B. A cytosolic chaperone complexes with dynamic membrane J-proteins and mobilizes a nonenveloped virus out of the endoplasmic reticulum. PLoS Pathog. 2014;10(3):e1004007. doi:10.1371/journal.ppat.1004007

285. Wang IH, Burckhardt CJ, Yakimovich A, Morf MK, Greber UF. The nuclear export factor CRM1 controls juxta-nuclear microtubule-dependent virus transport. J Cell Sci. 2017;130(13):2185-2195. doi:10.1242/jcs.203794

286. Wang M, Cao R, Zhang L, et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. Cell Res. 2020;30(3):269-271. doi:10.1038/s41422-020-0282-0

287. Wengler G. Cell-associated West Nile flavivirus is covered with E+pre-M protein heterodimers which are destroyed and reorganized by proteolytic cleavage during virus release. *J Virol*. 1989;63:2521-2526.

288. Wengler G. The regulation of disassembly of alphavirus cores. *Arch Virol*. 2009;154:381-390.

289. White J, Kielian M, Helenius A. Membrane fusion proteins of enveloped animal viruses. *Q Rev Biophys*. 1983;16:151-195.

290. Whittaker GR. Virus nuclear import. *Adv Drug Deliv Rev*. 2003;55:733-747.

291. Whittaker GR, Kann M, Helenius A. Viral entry into the nucleus. *Annu Rev Cell Dev Biol*. 2000;16:627-651.

292. Wickham TJ, Filardo EJ, Cheresh DA, et al. Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization. *J Cell Biol*. 1994;127:257-264.

293. Wickham TJ, Mathias P, Cheresh DA, et al. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell*. 1993;73: 309-319.

294. Wileman T. Aggresomes and pericentriolar sites of virus assembly: cellular defense or viral design? *Annu Rev Microbiol*. 2007;61:149-167.

295. Wolffe EJ, Weisberg AS, Moss B. Role for the vaccinia virus A36R outer envelope protein in the formation of virus-tipped actin-containing microvilli and cell-to-cell virus spread. *Virology*. 1998;244:20-26.

296. Xia X, Wu W, Cui Y, Roy P, Zhou ZH. Bluetongue virus capsid protein VP5 perforates membranes at low endosomal pH during viral entry. Nat Microbiol. 2021;6(11):1424-1432. doi:10.1038/s41564-021-00988-8

297. Xie J, Zhang P, Crite M, DiMaio D. Papillomaviruses Go Retro. Pathogens. 2020;9(4):267. doi:10.3390/pathogens9040267

298. Xu W, Santini PA, Sullivan JS, et al. HIV-1 evades virus-specific IgG2 and IgA responses by targeting systemic and intestinal B cells via

long-range intercellular conduits. Nat Immunol. 2009;10(9):1008-1017. doi:10.1038/ni.1753

299. Yamayoshi S, Yamashita Y, Li J, et al. Scavenger receptor B2 is a cellular receptor for enterovirus 71. Nat Med. 2009;15(7):798-801. doi:10.1038/nm.1992

300. Yeager CL, Ashmun RA, Williams RK, et al. Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature*. 1992;357:420-422.

301. Yuan P, Thompson TB, Wurzburg BA, et al. Structural studies of the parainfluenza virus 5 hemagglutinin-neuraminidase tetramer in complex with its receptor, sialyllactose. Structure (Camb). 2005;13:803-815.

302. Zhang P, Monteiro da Silva G, Deatherage C, Burd C, DiMaio D. Cell-penetrating peptide mediates intracellular membrane passage of human papillomavirus L2 protein to trigger retrograde trafficking. Cell. 2018;174(6):1465-1476.e13. doi: 10.1016/j.cell.2018.07.031

303. Zhang R, Kim AS, Fox JM, et al. Mxra8 is a receptor for multiple arthritogenic alphaviruses. Nature. 2018;557(7706):570-574. doi:10.1038/s41586-018-0121-3

304. Zhang Y, Bergelson JM. Adenovirus receptors. *J Virol*. 2005;79:12125-12131.

305. Zhao L, Imperiale MJ. Identification of Rab18 as an essential host factor for BK polyomavirus infection using a whole-genome RNA interference screen. mSphere. 2017;2(4):e00291-17. doi: 10.1128/mSphereDirect.00291-17

306. Zhao X, Zhang G, Liu S, et al. Human neonatal Fc receptor is the cellular uncoating receptor for enterovirus B. Cell. 2019;177(6):1553-1565.e16. doi: 10.1016/j.cell.2019.04.035

307. Zila V, Margiotta E, Turoňová B, et al. Cone-shaped HIV-1 capsids are transported through intact nuclear pores. Cell. 2021;184(4):1032-1046.e18. doi: 10.1016/j.cell.2021.01.025

308. Zvonarjev AY, Ghendon YZ. Influence of membrane (M) protein on influenza A virus virion transcriptase activity in vitro and its susceptibility to rimantadine. *J Virol*. 1980;33:583-586.

CHAPTER 5

Viral Replication Strategies

Sean P. J. Whelan

Viral genome diversity and replication strategies

Unique biology of virus replication

Subcellular sites of viral replication

Evasion of host response to infection

Error prone nature of RNA replication

Levels of segmentation

Host cell components for replication

Structures and organization of viral genomes

DNA vs RNA genomes

Single- and double-stranded genomes

Positive, negative, and ambisense genomes

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cis-Acting RNA signals and specificity

Satellite, dependent, and defective genomes

Expression and replication of DNA virus genomes

DNA virus genome strategies

Regulation of viral gene expression

Mechanisms of DNA replication and transcription

Latent and persistent infections

Viral oncogenes and neoplastic transformation

Expression and replication of RNA virus genomes

RNA virus genome strategies

Regulation of gene expression

Structural and nonstructural proteins

Host cell factors

Compartmentalization of replication sites

Mechanisms of RNA replication and transcription

Summary and perspectives

Acknowledgment

Replication of genetic information is the single most distinctive characteristic of living organisms, and nowhere in the biosphere is replication accomplished with greater economy and apparent simplicity than among viruses. To achieve the expression, replication, and spread of their genes, different virus families have evolved diverse genetic strategies and replicative cycles to exploit the biology of their hosts. Despite their comparatively limited genetic repertoire, viruses encode the information necessary to rewire their hosts to become viral factories. The intimacy of this relationship and the coevolution of virus and host continue to provide unique mechanistic insights into host biology at the molecular, cellular, organismal, and population levels. Understanding this interplay enriches our understanding of the biosphere in general and virus-host relationships in particular, but also creates opportunities for the rational development of antiviral drugs, and for domesticating viruses as expression vectors, live-attenuated vaccines, and pesticides. This chapter provides an overview of the replication strategies of the major virus families that infect vertebrates, attempting where possible to emphasize the general principles that guide and constrain virus replication and

evolution.

Viral Genome Diversity and Replication Strategies

Perhaps the most striking aspect of viruses at the molecular level is the diversity of their genome structures and replication strategies. Unlike cellular genomes, which consist uniformly of double-stranded DNA (dsDNA), viral genomes provide examples of almost every structural variation imaginable. As shown in Table 5.1, different families of viruses have genomes made of either double-stranded (ds) or single-stranded (ss) DNA or RNA; of either positive, negative, or ambisense polarity; of either linear or circular topology; and comprising either single or multiple segments. Each variation has consequences for the pathways of genome replication, viral gene expression, and virion assembly. This diversity argues strongly that viruses had several different evolutionary origins and can be thought of in D. J. McGeoch's evocative phrase as "mistletoe on the tree of life." As more sequences have been deposited, coupled with the recognition that only a small subset of those viruses are cultivatable, viral taxonomy has been updated. Accordingly, viruses are now classified in one of six realms the Adnaviria (filamentous dsDNA viruses of archaea), Duplodnaviria (dsDNA viruses), Monodnaviria (ssDNA viruses), Riboviria (RNA viruses), Ribozyviria (circular negative sense RNA genomes), and Varidnaviria (dsDNA that have jelly roll-like capsids). Taxonomy is further divided into 65 orders and 233 families,[128] the classifications of several vertebrate viruses are provided in Table 5.1. As more distant phylogenetic relationships and the number of genome sequences and protein structures increase, and as more powerful comparison algorithms become available, the boundaries between different classification groups fade revealing a continuum within several of the realms.

TABLE 5.1 Families and genera of viruses that infect vertebrates

Virus Family or Genera	Genome				Genome Replication	
	Type	Polarity[a]	Topology[b]	Segments	Enzyme	Intracellular site
Adenoviridae	dsDNA	Both	Linear	1	Viral DdDp	Nucleus
Anelloviridae	ssDNA	Negative	Circular	1	Cellular DdDp	Nucleus
Asfarviridae	dsDNA	Both	Linear	1	Viral DdDp	Cytoplasm
Circoviridae	ssDNA	Negative or ambisense	Circular	1	Cellular DdDp	Nucleus
Hepadnaviridae	dsDNA	Both	Linear	1	Virion RdDp	Nucleus/cytoplasm
Herpesviridae	dsDNA	Both	Linear	1	Viral DdDp	Nucleus
Iridoviridae	dsDNA	Both	Linear	1	Viral DdDp	Nucleus/cytoplasm
Papillomaviridae	dsDNA	Both	Circular	1	Cellular DdDp	Nucleus
Parvoviridae	ssDNA	Either	Linear	1	Cellular DdDp	Nucleus
Polyomaviridae	dsDNA	Both	Circular	1	Cellular DdDp	Nucleus
Poxviridae	dsDNA	Both	Linear	1	Viral DdDp	Cytoplasm
Arenaviridae	ssRNA	Ambisense	Linear	2	Virion RdRp	Cytoplasm
Arteriviridae	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm
Astroviridae	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm
Birnaviridae	dsRNA	Both	Linear	2	Virion RdRp	Cytoplasm
Bornaviridae	ssRNA	Negative	Linear	1	Virion RdRp	Nucleus
Bunyaviridae	ssRNA	Negative or ambisense	Linear	3	Virion RdRp	Cytoplasm
Caliciviridae	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm
Coronaviridae	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm
*Deltavirus*genus	ssRNA	Negative	Circular	1	RNA pol II	Nucleus
Filoviridae	ssRNA	Negative	Linear	1	Virion RdRp	Cytoplasm
Flaviviridae	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm
Hepeviridae	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm
Nodaviridae	ssRNA	Positive	Linear	2	Viral RdRp	Cytoplasm
Orthomyxoviridae	ssRNA	Negative	Linear	6-8	Virion RdRp	Nucleus
Paramyxoviridae	ssRNA	Negative	Linear	1	Virion RdRp	Cytoplasm
Picornaviridae	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm
Reoviridae	dsRNA	Both	Linear	10-12	Virion RdRp	Cytoplasm
Retroviridae	ssRNA	Positive	Linear	2 identical	Virion RdDp	Nucleus/cytoplasm
Rhabdoviridae	ssRNA	Negative	Linear	1	Virion RdRp	Cytoplasm
Togaviridae	ssRNA	Positive	Linear	1	Viral RdRp	Cytoplasm

[a]Polarity of the encapsidated genome.

[b]Topology of the encapsidated genome—note that some circularize during replication.

DdDp, DNA-dependent DNA polymerase; ds, double-stranded; RdDp, RNA-dependent RNA polymerase; ss, single-stranded.

Unique Biology of Virus Replication

As obligate intracellular parasites, all viruses depend heavily on functions provided by their host cells. This dependence, as well as the extensive metabolic overlap between host and parasite, limits the number of possible targets for antiviral therapy. Nevertheless, almost all viruses encode and express unique proteins, including enzymes, and many viruses exploit pathways of information transfer that are unknown elsewhere in the biosphere. This is particularly evident among the RNA viruses, which are the only organisms that are known to store their genetic information in the form of RNA. They accomplish this by replicating their genomes via one of two unique biochemical pathways—either by RNA-dependent RNA synthesis[104] (RNA replication) or, among the retroviruses, by RNA-dependent DNA synthesis (reverse transcription)[6,114] followed by DNA replication and transcription. Both pathways require enzymatic activities that are not usually found in uninfected host cells and must, therefore, be encoded by the viral genome and expressed during infection. Furthermore, in some families of RNA-containing viruses, those unique synthetic processes are required right at the start of the infectious cycle. This necessitates copackaging of the corresponding polymerase and other associated enzymes with the viral genome during the assembly of viral particles in preparation for the next round of infection. The

circular RNA genome of hepatitis delta virus encodes neither an RNA-dependent RNA polymerase nor a reverse transcriptase. Replication of this virus depends upon hepatitis B virus and is also dependent on the host DNA-dependent RNA polymerase II (reviewed in Ref.[113]).

Whatever the structure and replication strategy of their genomes, all viruses must express their genes as functional messenger RNAs (mRNAs) early in infection in order to direct the cellular translational machinery to make viral proteins. The various genomic strategies employed by viruses can, therefore, be organized around a simple conceptual framework centered on viral mRNA (Figs. 5.1 and 5.2). By convention, mRNA is defined as positive sense and its complement as negative sense. The pathways leading from genome to message vary widely among the different virus families and form the basis of viral taxonomy. Although it is generally believed that viruses originated from cellular organisms, perhaps fairly recently in evolutionary times, it remains possible that some RNA viruses are descended directly from a primordial "RNA world" or "ribonucleoprotein world," which may have predated the emergence of DNA and cells (reviewed in Ref.[8]).

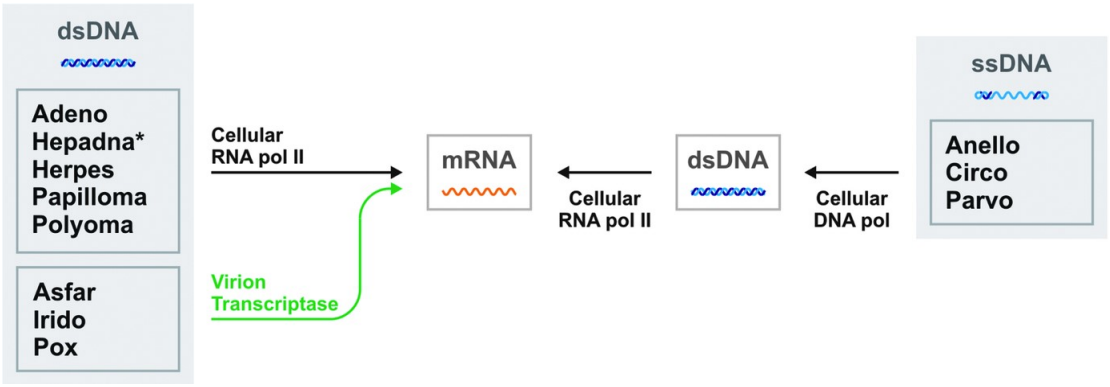

FIGURE 5.1 **Pathways of primary mRNA synthesis by DNA viruses of animals**. *Hepadnaviridae* replicate via reverse transcription of an ssRNA intermediate.

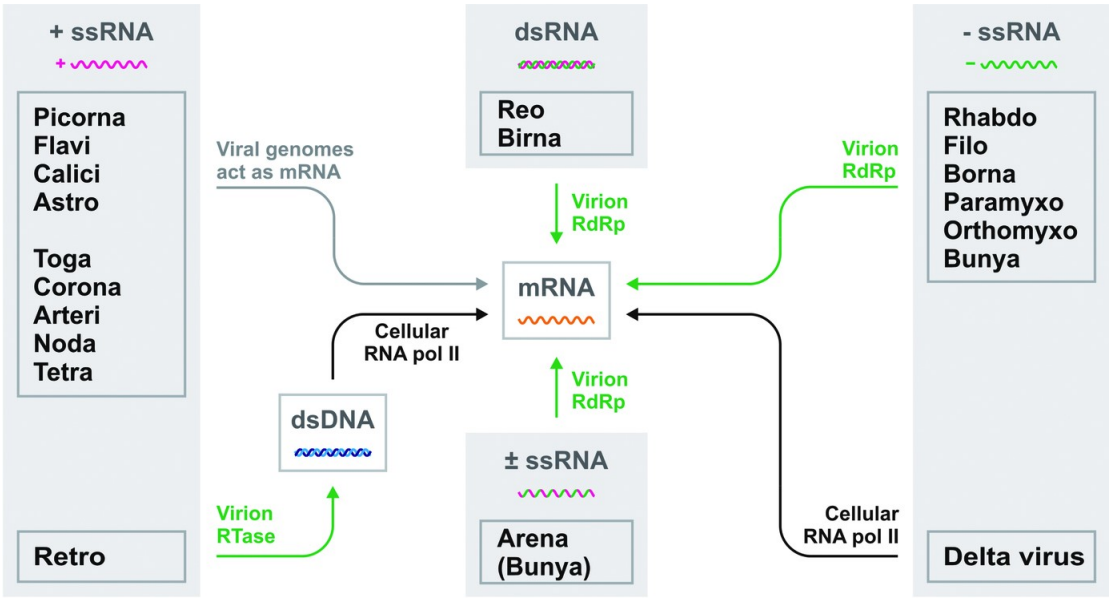

FIGURE 5.2 **Pathways of primary mRNA synthesis by RNA viruses of animals**. How RNA viruses produce mRNA at the start of infection depends upon the nature of the viral genome.

Subcellular Sites of Viral Replication

Most DNA viruses of eukaryotes transcribe and replicate their genomes and assemble progeny in the nucleus, the site of cellular DNA transcription and replication. The exceptions are the poxviruses, iridoviruses, and African swine fever virus, which replicate their DNA genomes partly or completely in the cytoplasm. In contrast, most RNA viruses replicate their genomes in the cytoplasm. However, in addition to the retroviruses that integrate DNA copies of their genomes into the host chromosomes, other notable exceptions to this generalization are the orthomyxoviruses, bornaviruses, and multiple plant-infecting rhabdoviruses, whose linear negative-sense RNA genomes replicate in the nucleus. The circular RNA genome of hepatitis delta virus (HDV) also replicates in the nucleus (Table 5.1). Each site of replication presents distinct opportunities and challenges in terms of which cellular components and pathways are available to be co-opted, and how the synthesis and trafficking of viral proteins, genome replication, virion assembly, and the release of progeny can be coordinated. For example, RNA splicing occurs only in the nucleus, so among the RNA viruses, this mechanism of accessing more than one open-reading frame in a single transcript can be employed by only the retro-, orthomyxo-, and bornaviruses that transcribe there. It is remarkable that the paramyxoviruses and filoviruses that replicate in the cytoplasm have evolved a transcriptional editing mechanism that achieves a similar result.[116,120] Irrespective of the site of replication (nuclear or cytoplasmic), the viral replication machinery itself is frequently compartmentalized within specific structures or viral-induced organelles. For example, herpesviruses form replication compartments within the nucleus at nuclear speckles.[16,101] Many RNA viruses that replicate in the cytoplasm do so in association with membranes or membrane-less inclusionlike structures that contain the viral replication

machinery[26,68] and share properties with phase-separated compartments.[55]

Evasion of Host Response to Infection

To ensure their survival, host organisms have evolved a variety of responses to combat viral infection. In turn, many viruses express specific gene products that act to circumvent one or more of those antiviral defense mechanisms. Examination of these measures and countermeasures provides a revealing glimpse into the heart of the host-parasite relationship as it plays out in nature. Host-defense mechanisms can be categorized as innate or adaptive. Among the former, which operate at the cellular level, are apoptosis, necroptosis, and pyroptosis (three distinctive cell death responses to virus infection that limit the spread of infection), the induction and action of interferons in vertebrates (inducible cytokines that render cells resistant to infection by inducing a multifaceted antiviral state), and RNA interference in plants and invertebrates (a sequence-specific mechanism of RNA degradation). Adaptive immune mechanisms operate at the organismal level and include the cell- and antibody-mediated immune response (see Chapter 10). Specific restriction factors have been identified that limit the replication of subsets of viruses. Such factors include tripartite motif containing protein 5 (TRIM5), which binds the incoming human immunodeficiency virus type 1 (HIV-1) capsid and through multiple mechanisms limits the establishment of infection (reviewed in Ref.[39]). The apolipoprotein B mRNA editing, enzyme catalytic (APOBEC) family, which induce biased hypermutation in RNA through a cytidine deaminase activity that converts C to U (reviewed in Ref.[126]). Bone marrow stromal antigen 2 (Bst2)/tetherin is incorporated into the membranes of some enveloped viruses resulting in a linking together of budding viral particles. Multiple other cellular proteins have been termed "restriction factors," the expression of most of which are stimulated by interferon (reviewed in Ref.[106]). In different viruses, mechanisms and gene products have been identified that inhibit apoptosis, intercept interferons or suppress their activities, obstruct RNA interference, either evade or suppress different arms of the adaptive immune response, or block intrinsic restriction factors.[5,38,52,53,70,79,103,107,108,117]

Viruses are sensed by the host in ways that appear to involve recognition of unique signatures present in viral genomes or gene products. Such signatures are termed pathogen-associated molecular patterns (or PAMPs) and are recognized by an array of host pathogen-recognition receptors (or PRRs). Those PRRs include the toll-like receptors (TLRs), which are membrane-associated molecules that sense invading pathogens directly at the plasma membrane or during endosomal transit. The retinoic acid inducible gene (RIG)-like receptors (RLRs), which are cytoplasmic RNA helicases that recognize the products of RNA viral nucleic-acid replication, and the absent in melanoma 2-like receptors that recognize cytoplasmic DNA. The nucleotide binding domain (NBD) leucine-rich repeat (LRR) containing receptor (NLR) family members sense bacterial peptidoglycan and some viruses. A striking example of such PRR function is the detection of off-pathway products of replication such as abortive initiation products, dsRNA, and defective viral genomes, which can serve as ligands for the cytoplasmic sensors RIG-I and melanoma differentiation association protein 5 (MDA-5) to engage in a signaling cascade that leads to the activation of interferon.[43] The net result of interferon activation is both the blocking of infection within the cell, and the preactivation of defense mechanisms in neighboring cells to render them less susceptible to infection. The latter is accomplished by the transcription of interferon (IFN)-stimulated genes (ISG), which themselves act to block various steps in the replication cycle of DNA and RNA viruses.[106] In turn, viruses themselves have evolved countermeasures to such host-defense mechanisms that act to block the induction of IFN itself or to interfere with specific ISG function.[22,46] The elaborate arms race between viruses and their hosts is described in more detail within the specific chapters dealing with individual virus families. Molecular signatures of this arms race throughout evolution are also visible in the sequences of virus and host genes (reviewed in Ref.[115]). Retroviruses provide a unique insight into this, since they integrate into the host genome. Evidence for integration of portions of other viral genomes into the host chromosome including RNA viruses such as bornavirus, and lymphocytic choriomeningitis virus, has also emerged.[35,90]

Error Prone Nature of RNA Replication

The polymerases that catalyze RNA replication and reverse transcription have minimal proofreading activities. The polymerase error rate of such RNA-dependent RNA polymerases (RdRps) and reverse transcriptases is approximately three orders of magnitude higher than that of DNA-dependent DNA polymerases and approaches the reciprocal of their genome length.[29,60,82] The net result is that the genomes of RNA viruses evolve at a much faster rate than those of their hosts. Biologically, RNA viruses, therefore, represent a swarm of sequences around a consensus sequence or master sequence.[33,69] This molecular swarm provides a fertile source of phenotypic variants that can respond rapidly to changing selection pressures by shifting its composition. As a consequence, RNA viruses can evolve up to 1 million times faster than DNA-based organisms. The error prone nature of RNA virus replication is also critical for pathogenesis in infected hosts. The diversity of viral sequences regenerated following bottleneck transmission of HIV in humans,[102] and experimental poliovirus infection of mice[94,118] provide striking examples of this *in vivo*. In the case of HIV, the resulting sequence variation achieved following transmission of a limited number of genomes is enormous and accounts for—among other phenotypes—the rapid escape of the virus from neutralizing antibody and the escape from antiviral monotherapy.

Such rapid rates of evolution are not without cost for the RNA viruses however, because higher polymerase error rates impose upper limits on genome size. The combination of replicative error rate and genome size defines an "error threshold" above which a virus cannot maintain even the sequence integrity of its quasispecies.[33] As a result, few RNA virus genomes contain more than 30 kilobases (kb) and most have between 5 and 15 kb. RNA genomes of this size are poised just below their error thresholds, and although their genetic diversity inevitably wastes individual progeny that carry deleterious mutations, the cost is offset by the potential for rapid evolutionary response to changing selective pressures. This positioning of RNA viruses—just below their error threshold—may also present an opportunity for antiviral development. Specifically, therapeutics that lead to an increase in error rate can shift the balance beyond the error threshold toward "error catastrophe." Indeed evidence has accumulated that this is one such mechanism by which ribavirin, an adenosine analog, may inhibit the replication of some RNA viruses.[21] The largest RNA virus genomes currently recognized are those of the coronaviruses, which are ~30 kb. Strikingly, for coronaviruses, the nonstructural protein nsp14 functions as a 3′-5′ RNA exonuclease that corrects errors introduced by the polymerase during replication.[28,32] Despite the presence of this proofreading mechanism, which could help maintain genome integrity, replication of coronaviruses is error prone.[28] The 3′-5′ RNA exonuclease activity also mediates RNA recombination during replication that likely also aids in maintaining genome integrity.[48]

Levels of Segmentation

Another distinctive feature of eukaryotic cells—besides their partitioning into nuclear and cytoplasmic compartments—has a profound influence on the biology of their viruses. On most mRNAs, eukaryotic ribosomes require a methylated mRNA cap structure at the 5′ end that plays a critical role in signaling the initiation of protein synthesis. As a result, eukaryotes typically conform to the "one mRNA one polypeptide chain" rule; with very few exceptions, each message operates as a single translational unit. Similarly, viral RdRps generally appear somewhat restricted in their ability to access internal promoter elements on RNA templates, and this creates a problem of how an RNA virus can derive several separate protein products from a single genome.

Through evolution, different RNA virus families have found three different solutions: fragmentation at the level of proteins, mRNAs, or genes, with some viruses using more than one of those solutions. For example, RNA viruses in the picorna-, toga-, flavi-, and retrovirus families rely on extensive proteolytic processing of polyprotein precursors to derive their final protein products.[30] Others (in the orders *Mononegavirales* and *Nidovirales*) depend on complex transcriptional mechanisms to produce several monocistronic mRNAs from a single RNA template.[2,105] Still others (in the reo-, orthomyxo-, bunya-, and arenavirus families, among others) have solved the problem by fragmenting their genomes and assembling virions that contain multiple genome segments, each often representing a single gene.[36,78,84] Among plant viruses, such RNA genome segments are often packaged into separate virions, necessitating coinfection by several virus particles to transmit infectivity,[127] but the genome segments of animal viruses are typically copackaged into single virions. In contrast, DNA viruses seldom use either genome segmentation or polyprotein processing. This is likely due to the relative ease with which monocistronic mRNAs can be transcribed from internal promoter elements of dsDNA, and the extensive use of differential splicing of nuclear transcripts to express promoter-distal open-reading frames.

Host Cell Components for Replication

Viruses depend on their host cells to support their replication, and this degree of dependency—to some extent—reflects their genome size. All viruses depend on the host translational machinery, large DNA viruses, such as mimivirus may encode specific initiation factors that may provide a translational advantage for viral genes.[18] Entry of viruses into cells usually requires specific host-cell factors and often involves co-opting of cellular endocytic pathways.[80] The end point of entry is the release of the minimal viral replication machinery into the host-cell cytoplasm to initiate infection. How viruses establish infection in the hostile environment of the host cell remains one of the least understood steps of the viral replication cycle. The input genomes must either associate directly with ribosomes, in the case of positive-strand RNA viruses, or be copied into mRNA, in the case of the negative-strand RNA viruses, dsRNA viruses, and DNA viruses. Because the particle-to-infectivity ratio of some viruses approaches 1:1, this process must be highly efficient despite its inherent challenges. Our knowledge of the subsequent viral rewiring of host-cell structures to establish replication compartments, traffic viral proteins and nucleic acids, and assemble viral particles is far from complete, but has yielded a wealth of information into host biology as well as that of the viruses themselves. Indeed, study of viruses has contributed enormously to our understanding of promoters, transcriptional enhancers, the mRNA cap structure, RNA splicing, and mechanism of translation. Similarly, critical discoveries in host-cell transport and trafficking pathways including endocytosis, exocytosis, and secretory transport were achieved because of the ability to synchronize infections with viruses. System approaches including RNA interference (RNAi), proteomics, gene-knockout studies, particularly those capitalizing on CRISPR-Cas systems (clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein [Cas]), and microarrays are helping to further transform our understanding of the virus-host interaction at the molecular level. Despite the explosion of progress in the last decade, we have yet to understand fully the complexities of the interactions of any virus with its host. Zoonotic viruses must strike a balance for optimal replication in often quite disparate hosts, likely adding further complexity to this intimate relationship. Striking examples of this are provided by members of the *Flaviviridae*, such as Dengue virus (which replicates in both its mosquito host and animals), and experimentally with many viruses including vesicular stomatitis virus (which replicates in virtually all eukaryotic cells in culture).

Structures and Organization of Viral Genomes

DNA vs RNA Genomes

Among families of viruses that infect vertebrates, those with RNA genomes outnumber those with DNA genomes by about 2 to 1 (Table 5.1); among viruses infecting plants, the disparity is even greater. Indeed, no dsDNA viruses of plants are known except for those that like the hepadnaviruses of vertebrates, replicate via reverse transcription. This remarkable observation remains to be explained, but it may suggest that non-RT dsDNA viruses arose only after animals and plants diverged. Be that as it may, the prevalence of RNA viruses attests to the evolutionary success and versatility of RNA as genetic material for smaller genomes. As discussed previously, the high error rates of RNA replication restrict RNA genome sizes to ~30 kb or less, whereas proofreading and error repair ensure sufficiently accurate replication of DNA virus genomes as large as that of the 1200-kb megaviruses and the 2400-kb pandoraviruses.[4] In addition, the fact that DNA is more chemically stable than RNA likely explains why all known viruses of thermophilic hosts have dsDNA genomes.

Single- and Double-Stranded Genomes

Although all viral genomes replicate via conventional Watson-Crick base pairing between complementary template and daughter strands, viruses that belong to different families encapsidate and transmit different molecular stages of the genome replication cycle. Families of ssRNA viruses outnumber families of dsRNA viruses by almost 10 to 1, roughly the inverse of the ratio between ssDNA and dsDNA viruses. In view of the greater chemical stability of double-stranded nucleic acids of both types, this difference calls for an explanation. Two possibilities seem plausible: First, dsRNA viruses must somehow circumvent the translational suppression that can result from the coexistence of equimolar amounts of the sense and antisense RNAs. How the dsRNA reoviruses solve this problem is addressed in Chapter 11, Volume 3. Second, dsRNA is widely recognized by the cells of higher eukaryotes as a signal for the induction of defense mechanisms that act to suppress viral replication, such as the IFN system in vertebrates (see also Chapter 9), gene silencing in plants, and RNAi in a variety of organisms.[15,43,108,125] These effects probably suffice to explain the relative scarcity of dsRNA virus families.

For these same reasons, it is important even for ssRNA viruses to limit the accumulation of replicative intermediates that contain regions of dsRNA, and the strategies to ensure this differs between the positive- and negative-sense RNA viruses. All known positive-strand RNA viruses synthesize disproportionately low amounts of the negative-strand RNA—typically 1%-5% of the levels of the positive strand—and thereby minimize the potential for dsRNA accumulation. Moreover, because the replication of these viruses appears to universally occur in sequestered

membranous compartments, there appears to be a physical separation of the replicative intermediates from the host-cell cytoplasm, likely reducing the chances of detection.[26] In contrast, negative-strand RNA viruses, which need substantial amounts of both positive- and negative-sense RNAs to use as messages and progeny genomes, respectively, prevent the complementary RNAs from annealing to one another by encasing the genomic and antigenomic RNAs with a viral nucleocapsid protein that sequesters the RNA and impedes base pairing.[3,47] Here, RNA synthesis also appears confined at some stages of infection to specific subcellular compartments that may help serve to limit detection of viral products of RNA synthesis by the innate immune system.

Positive, Negative, and Ambisense Genomes

The differences between positive- and negative-strand RNA viruses extend beyond the polarity of the RNA assembled into virions. Positive-sense RNA genomes exchange their virion proteins for ribosomes and cellular RNA binding proteins at the onset of infection. Once synthesized and assembled, the virus-specified RdRp and other nonstructural proteins replace the ribosomes to accomplish RNA replication. Virion structural proteins are reacquired during the assembly of progeny virions. In contrast, negative-strand RNA genomes and their antigenomic complements remain associated with their nucleocapsid proteins, both within the viral particles and throughout the viral replication cycle, even during RNA replication. These fundamentally different adaptations can be attributed to the fact that whereas positive-sense RNA genomes must satisfy criteria for translation that are dictated by the host cell, negative-sense RNA genomes must only satisfy the template requirements for the virus-specified RdRp because they are replicated but never translated. Although the precise mechanism by which the protein-coated templates of negative-strand RNA genomes are copied by their cognate polymerases is not fully understood, short naked RNAs that correspond to the terminal promoters can be copied by their viral polymerases.[27,66,81] Such experimental evidence is consistent with a model for RNA synthesis in which the nucleocapsid protein is transiently displaced from the template RNA during copying of the genome. Structural studies of the polymerases of multiple negative strand RNA viruses (reviewed in Ref.[99]) suggest that transient dissociation of the template RNA from 2 to 3 molecules of the template N protein would be sufficient to facilitate the copying of the genomic RNA. Precisely how such displacement is achieved is uncertain.

The dsRNA virus genomes are intermediates between the two. The parental genome remains sequestered within a subviral particle during the synthesis of the unencapsidated positive-sense mRNA transcripts, which are replicated to produce progeny dsRNAs only after being assembled into subviral core particles.[91] Although the core RdRps of each of these viruses are structurally as well as functionally analogous, the distinctions in the genomic structure likely place additional structural constraints on the viral polymerase complexes.

Linear and Circular Genomes

Genome replication not only requires an acceptable error rate as described previously but must also avoid the systematic deletion or addition of nucleotides. Genome termini are particularly troublesome in this respect, a fact that has been dubbed “the end problem.” For DNA replication, the end problem is exacerbated by the fact that DNA polymerases cannot initiate the synthesis of daughter strands and must, therefore, use primers, thus creating additional complications of replicating the primer-binding site(s). Among several known solutions, the most economical and widespread in nature is to eliminate the ends altogether by covalently circularizing the genomic DNA, as occurs in the genomes of prokaryotes. Polyoma-, papilloma-, circo-, and anellovirus genomes follow this model, and the dsDNA genomes of herpes and hepadnaviruses, although linear, in virions are covalently circularized before replication. Poxviruses and asfiviruses also have linear dsDNA genomes, but in these cases, the individual complementary strands are covalently continuous at the termini of the duplex, which provides another solution to the end problem. A similar close-ended duplex DNA is generated during the replication of the ssDNA genomes of parvoviruses (Chapter 6, Volume 2). Terminal redundancy (iridoviruses), inverted terminal repeats (adenoviruses), and the use of protein primers that do not occlude the binding site (adenoviruses and hepadnaviruses) represent the other ways that DNA viruses have evolved to ensure accurate and complete replication of their genome termini.

Unlike DNA polymerases, most RNA polymerases do not require primers, so RNA genomes are less susceptible to the end problem. Accordingly, most RNA genomes are linear molecules. Covalently closed circular RNAs are found only in HDV in animals (Table 5.1) as well as among the viroids and other subviral RNA pathogens that infect plants. Nevertheless, the termini of linear RNA genomes are vulnerable to degradation, and their replication is likely to be particularly error prone. Consequently, every family of RNA viruses has features designed to preserve the termini of the genome.[7] For example, many positive-strand RNA viruses have a 5′ cap structure and 3′ polyadenylate tail that serve to protect eukaryotic RNAs against degradation, and a similar role is likely played by the VPg that is covalently linked to the 5′ end of the picornavirus genomes,[71] and by the stable RNA secondary structures present at the 3′ end of the flaviviral RNA and other genomes. The 3′ ends of many plant virus RNAs form clover leaf structures that resemble transfer RNAs (tRNAs) so closely that they are recognized by the cellular tRNA charging and modifying enzymes.[31] In addition to playing protective roles, terminal modifications of positive-sense RNAs may also serve to bring their ends together by binding to interacting cellular proteins such as the poly(A) binding protein and cap-binding complex, thereby forming noncovalent functionally circular complexes that may promote repetitive translation by ribosomes and repetitive replication by RdRps.[56,119]

Unlike the genomes of positive-sense RNA viruses, negative-sense and ambisense RNA virus genomes lack covalent terminal modifications. Those RNA genomes are, however, present as a ribonucleoprotein complex in which the genomic and antigenomic RNAs are found encapsidated by the viral nucleocapsid protein. Terminal *de novo* initiation during replication requires a priming loop present within those polymerases that stabilizes the initiation complex, and this is distinct from transcription of the viral genome. For the nonsegmented negative-sense RNA viruses, subsequent *de novo* initiation at internal gene-start sequences of the genome is not dependent upon the key priming loop residues suggesting a different mechanism of initiation. For the segmented negative-sense and ambisense viruses, mRNA transcription is initiated by short capped mRNA primers cannibalized from host cell mRNA either in the form of pre-mRNA in the nucleus or mature mRNA in the cytoplasm. In other solutions to the end problem among the RNA viruses, retroviral genomes are terminally redundant and have direct repeats of 12-235 nucleotides at each end that maintain and restore the integrity of the termini during reverse transcription and virus replication.

Segmented and Nonsegmented Genomes

As discussed previously, segmentation of RNA genomes is one way to facilitate the production of multiple gene products in eukaryotic cells, but it also means that the various segments must each contain appropriate *cis*-acting signals to mediate their expression, replication, and assembly

into virions. In some virus families whose members have segmented genomes (eg, the orthomyxoviruses and some reoviruses), these signals comprise conserved sequences at the RNA termini, but in others (eg, the bipartite nodaviruses and tetraviruses), sequence conservation between the segments is minimal. In these latter cases, the specificity of RNA replication and assembly is presumably dictated by conserved RNA secondary or tertiary structures. Moreover, segmentation of the viral genome requires a level of coordination to ensure that the correct amounts of viral gene products are expressed and to ensure the packaging of multiple genome segments to form infectious virus particles. Furthermore, in the case of the negative-sense, ambisense, and dsRNA viruses that have segmented genomes, a mechanism is required to ensure that the polymerase is packaged into the virus particle so that the incoming segments can be transcribed into mRNA. For the dsRNA viruses, the polymerase is an integral structural component of the core transcribing particle ensuring that the polymerase and capping machinery are present within the incoming particle. Each segment of influenza virus contains the heterotrimeric polymerase complex in complex with the genomic termini. In the case of the arenavirus, Machupo, the small viral protein Z, locks the polymerase on the promoter in an inactive form.[67]

Evidently, the evolutionary barrier between viruses with segmented and nonsegmented RNA genomes is readily transversed because both genome types occur in members of the alphavirus-like supergroup, a taxonomic cluster based on phylogenetic comparisons of nonstructural protein sequences. Indeed, among the tetraviruses, segmented and nonsegmented genomes can even be found in the same family. Furthermore, the genomes of some togaviruses, rhabdoviruses, and paramyxoviruses, which are naturally nonsegmented, have been experimentally divided into segmented genomes without destroying viral infectivity,[40,111] thus confirming the flexibility of RNA genomes in this regard. Nevertheless, genome segmentation has major effects on the biology of a virus because individual segments can reassort between dissimilar strains in coinfected cells, which enables segmented genome viruses to make substantial evolutionary leaps by horizontal gene transfer. This mechanism underlies the antigenic shifts that produce new pandemic strains of the orthomyxovirus influenza virus (see Volume 1, Chapters 14 and 15).

As discussed previously, genome segmentation is almost unknown among DNA viruses, most likely because internal initiation of transcription and alternative splicing provide more facile ways to access multiple open-reading frames. Only the polydnaviruses, a family of dsDNA viruses that infect parasitic wasps and participate in a complex and unusual host-parasite relationship, show extensive DNA segmentation.[7]

cis-ACTING RNA SIGNALS AND SPECIFICITY

Replication and packaging of viral RNAs display striking specificity; both processes unerringly pick the correct viral molecules from among thousands of cellular RNAs that may be much more abundant. This is generally attributed to the presence of *cis*-acting signals that selectively channel the viral RNAs into replication and assembly complexes, but for some RNA virus genomes, these signals remain to be clearly identified. *cis*-Acting signals that have been characterized comprise in addition to linear nucleotide stretches, RNA secondary structures such as bulged stem-loops, tRNA-like cloverleaves, and pseudoknots that create distinctive three-dimensional molecular shapes that interact specifically with the viral enzymes and structural proteins. Although high-resolution structures have been determined for many RdRps, reverse transcriptases, and viral capsids, our understanding of the molecular basis of specificity in RNA replication and virus assembly is limited by the knowledge of the three-dimensional structures of viral RNA and its *cis*-acting signals at the particular stages of replication or assembly. The structural basis of RNA specificity during replication and assembly has often proved elusive, perhaps because the specificity determinants can be redundant, dispersed, or global properties of the viral genome. Furthermore, in both RNA replication and assembly, specific interaction is often followed by less-specific RNA-protein interactions that propagate the reactions. The transitions between these different stages are beginning to be explored including polymerase structures in complex with the templates at various stages of synthesis or recognition of RNA by portions of viral capsid proteins. Where structures have been attained, noncanonical base pairing in the RNA in the presence of the viral polymerase as exemplified by influenza A virus (reviewed in Ref.[121]) or the coat protein of alfalfa mosaic virus[50] can play a key role in regulating replication and assembly. Much still remains to be learned concerning the recognition of *cis*-acting RNA signals and how they promulgate RNA replication and assembly.

Our understanding of *cis*-acting regions of RNA viral genomes has also benefitted from the application of a chemical probing methodology termed selective 2′ hydroxyl acetylation analyzed by primer extension (SHAPE), first applied to provide an overview of the complete genome of HIV-1.[122] In addition to correctly identifying known structures within the HIV-1 genome, several structures were identified within the coding regions at regions close to the positions of polyprotein processing. Such structured elements are thought to lead to a slowing of ribosomes to facilitate the correct folding of the preceding region of the polypeptide chain. Application of this methodology is helping to understand the *cis*-acting elements within RNA virus genomes as well as the overall structure of viral genomes, and the discovery of more processive reverse transcriptases that allow accurate probing of longer and more structured RNAs will likely further enhance the utility of this approach.[49]

Satellite, Dependent, and Defective Genomes

Occasionally, subviral genomes arise that are neither independently infectious nor essential for infectivity, but nevertheless contain *cis*-acting signals that promote their own replication and/or packaging by the proteins encoded by another virus. Such satellite nucleic acids are parasitic on the parental virus and can modulate its replication and virulence.[109] Most commonly, they are ssRNAs, but dsRNA and ssDNA satellites are also known. Among the RNA viruses of animals, a prime example is hepatitis delta virus (or HDV), which packages its ssRNA genome in virion proteins encoded by the hepadnavirus hepatitis B virus and can severely exacerbate its pathogenicity.[112,113] Dependence of an RNA satellite on a DNA virus parent is unusual; more commonly satellite, RNAs are replicated and encapsidated by the proteins of an RNA virus parent with which they share at least some sequence homology. In some instances, satellite RNAs encode their own distinct capsid proteins, or proteins required for RNA replication (as in the case of HDV), but in others they are translationally silent. Satellite RNAs are much more common among the viruses of plants than those of animals, perhaps because the transmission of animal viruses between hosts generally involves narrower bottlenecks that select against the spread of satellites. Dependence of one virus on another is also found occasionally among viruses with DNA genomes. For example, adeno-associated virus (family *Parvoviridae*, genus *Dependoparvovirus*) requires coinfection of host cells by adenoviruses or herpesvirus to provide helper functions necessary for its replication.

In contrast to the transmission of viral infection between hosts, the spread of infection within a single animal usually involves successive episodes of localized viral replication. These conditions favor the generation and amplification of defective viral genomes, which can arise from a simple internal deletion of genes as well as more complex genome rearrangements that occur during RNA replication. Like satellite RNAs, defective RNAs parasitize the parent virus and usually interfere with its replication, but because they also depend upon it for their own survival, they typically establish a fluctuating coexistence. Most families of animal RNA viruses readily generate defective interfering (DI) RNAs in cell culture, but their influence on viral disease and evolution is less well understood (reviewed in Ref.[42]).

Expression and Replication of DNA Virus Genomes

DNA Virus Genome Strategies

Viral DNA genomes range in size from the 1.8-kb circoviruses to the 2400-kb genomes of the pandoraviruses.[4,95] This vast difference in the coding capacity means that viruses from different families vary widely in how many of the functions necessary for viral replication they encode themselves. For example, DNA viruses with small genomes such as the polyoma-, papilloma-, and parvoviruses use host-cell enzymes for transcription and replication (Figs. 5.3 and 5.4). Those with intermediate-size genomes (up to 35 kb) such as adenoviruses encode much of their DNA replication machinery including a DNA polymerase, terminal protein, and ssDNA binding protein, but they employ cellular RNA polymerase II and III for transcription (Fig. 5.5). Those with larger genomes (150-350 kb), such as the herpesviruses and poxviruses, encode DNA polymerases and binding proteins. In the case of herpesviruses, multiple specific transcription factors serially modify the promoter specificity of RNA polymerase II (Fig. 5.6), or in the case of the poxviruses multi-subunit transcriptase complexes perform all the functions of mRNA capping and polyadenylation as well as RNA transcription (Fig. 5.7). Hepadnaviruses buck this general trend in that they are small genomes (3 kb) but encode the DNA polymerase/reverse transcriptase that executes their unique mechanism of DNA replication via an ssRNA intermediate (Fig. 5.8).

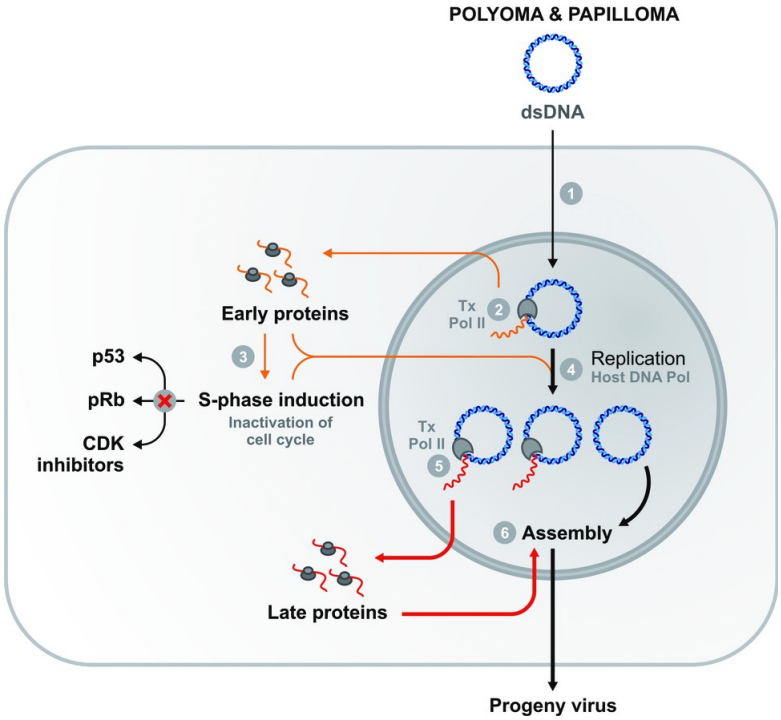

FIGURE 5.3 **Simplified view of the replication scheme of *Polyomaviridae* and *Papillomaviridae*.** The circular dsDNA genome is transported to the nucleus (*step 1*) where cellular RNA polymerase II transcribes the early genes (*step 2*) that encode the viral oncoproteins or transforming (T) antigens. The products of *step 2* interfere with the host-cell cycle control proteins p53 and pRb or interact with inhibitors of cyclin-dependent kinases (CDKs) to stimulate cellular DNA replication (*step 3*). In nonpermissive cells that cannot support the vegetative replication cycles and, therefore, survive the infection, these early events can lead to neoplastic transformation. In permissive cells, the viral DNA is replicated by the host-cell DNA polymerase (*step 4*), following which cellular RNA pol II can transcribe the late genes that encode viral structural proteins (*step 5*). The assembly of viral particles occurs in the nucleus (*step 6*). The thickness and color intensity of the *arrows* signifies the predominant events.

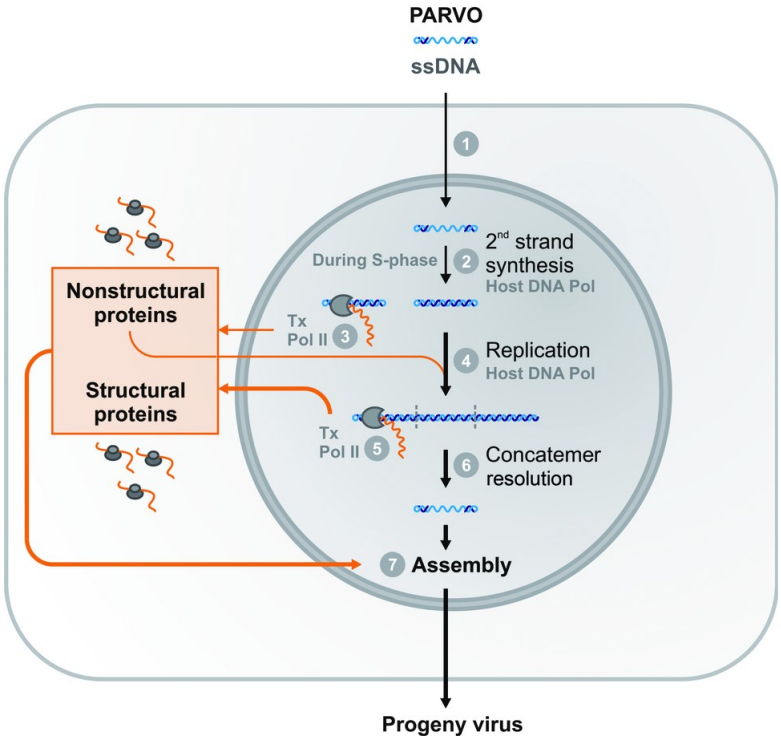

FIGURE 5.4 **Simplified view of the replication scheme of *Parvoviridae***. Following entry, the linear ssDNA genome is delivered to the nucleus (*step 1*) where self-primed second strand synthesis is mediated by the host DNA polymerase during the S phase of the cell cycle (*step 2*). The resulting dsDNA hairpin is transcribed by the cellular RNA polymerase II (*step 3*) to produce mRNAs that encode viral nonstructural and structural proteins and is ligated to form a covalently continuous duplex. The nonstructural proteins promote further DNA replication by the host DNA polymerase, which occurs via a rolling hairpin mechanism to produce double-stranded concatemers of the viral genome (*step 4*). The concatamers are templates for transcription by host RNA polymerase II to produce further viral proteins (*step 5*), and they are resolved (*step 6*) prior to assembly (*step 7*) into viral particles.

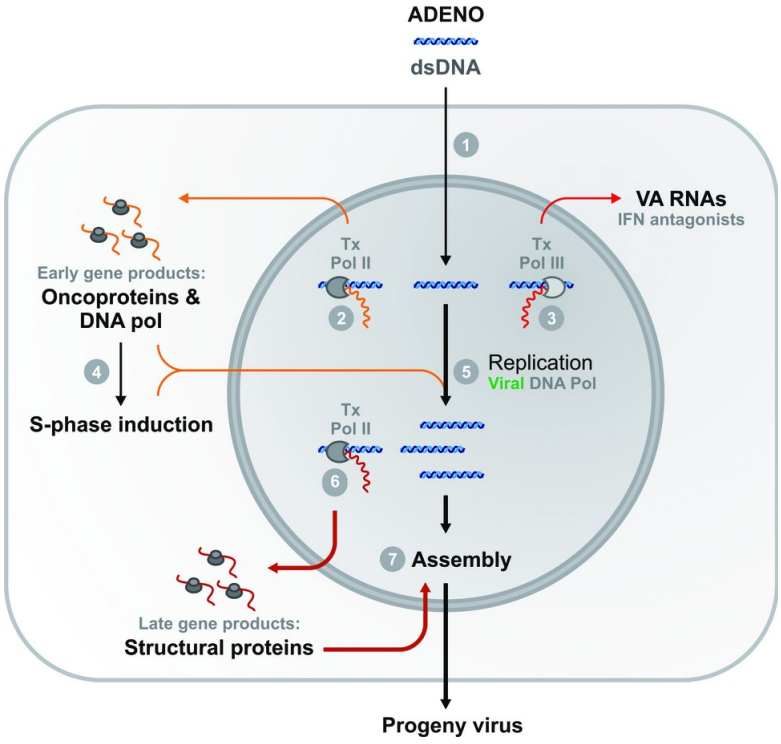

FIGURE 5.5 **Simplified overview of the replication scheme of *Adenoviridae***. The linear dsDNA genome is delivered into the nucleus (*step 1*), where it is transcribed by the host cell RNA polymerase II (*step 2*) to produce the early gene products including oncoproteins and the viral DNA polymerase. In adenoviruses of primates, the host RNA polymerase III also transcribes the genome to produce VA RNAs that act as interferon antagonists (*step 3*). The early gene products override cell cycle controls and inhibit apoptosis (*step 4*) as well as provide the essential viral polymerase components for genome replication (*step 5*). Following DNA replication, the late genes are transcribed by the host RNA polymerase II from a single major late promoter, and following extensive differential splicing provide the viral structural proteins (*step 6*). Virus assembly (*step 7*) occurs in the nucleus.

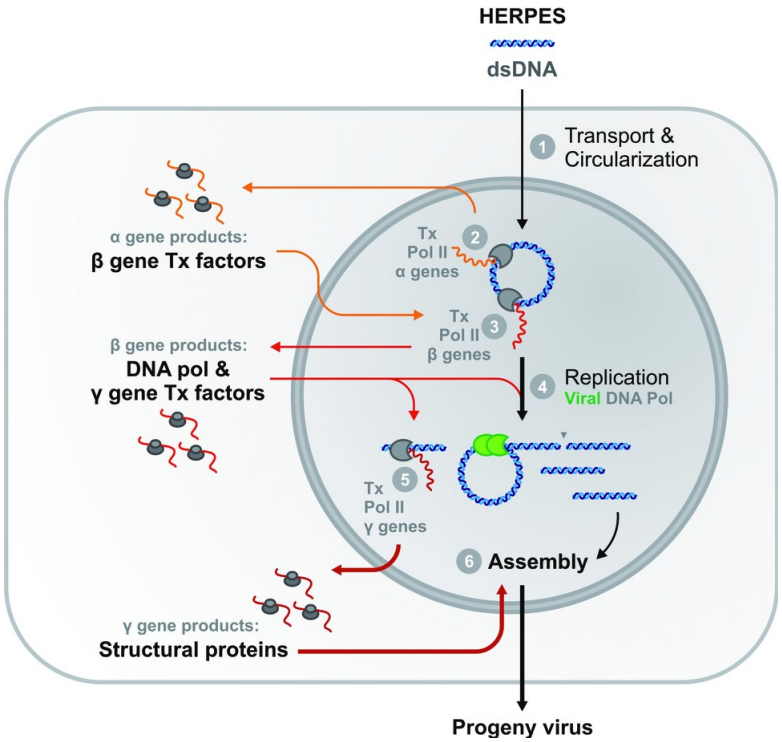

FIGURE 5.6 **Simplified overview of the replication scheme of *Herpesviridae***. The linear genomic dsDNA genome is delivered to the nucleus, where it undergoes covalent circularization (*step 1*). Cellular RNA polymerase II transcribes the immediate-early *α* genes (*step 2*) that encode transcription factors that mediate the host RNA polymerase II recognition of the promoters for the delayed-early *β* genes (*step 3*). Those genes encode the viral DNA polymerase and other proteins required for genome replication (*step 4*) as well as transcription factors required for the pol II–mediated expression of the late *γ* genes that encode most of the structural proteins (*step 5*). Virions assemble in the nucleus (*step 6*) and exit through the nuclear pore. The assembled virion contains the necessary transcription factors for expression of the immediate-early *α* genes on infection of the next cell. Note that this scheme represents only the vegetative cycle of *Herpesviridae* replication. Readers are referred to the individual *Herpesviridae* chapters regarding the establishment, maintenance of, and reactivation from latency.

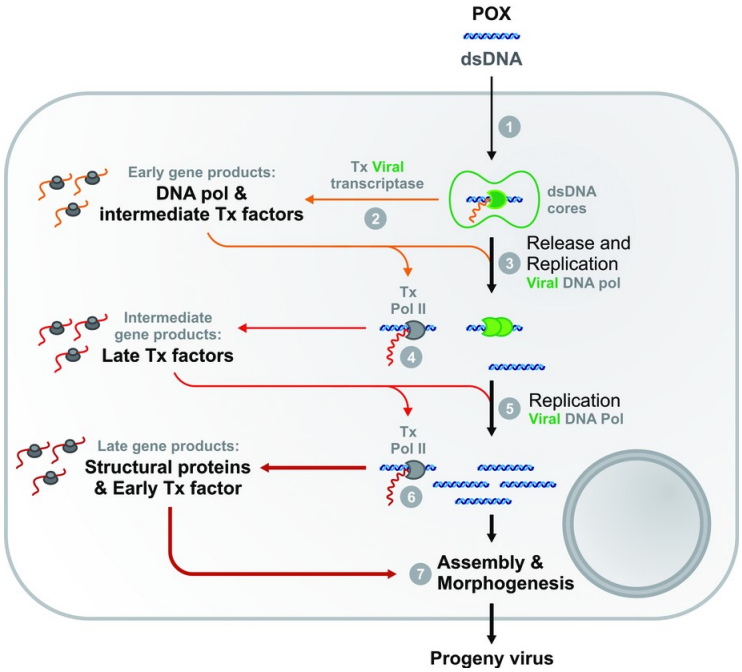

FIGURE 5.7 **Simplified scheme of the replication of *Poxviridae***. Entry delivers the viral core containing the dsDNA genome into the cytoplasm—the site of viral RNA synthesis (*step 1*). In the cytoplasm, the multisubunit virion DNA-dependent RNA polymerase transcribes the early viral genes (*step 2*), which comprise ~50% of the genome. The early gene products include factors that mediate the release of the dsDNA into the cytoplasm (second stage uncoating), the viral DNA polymerase and associated enzymes required for replication (*step 3*), and transcription factors that direct the viral RNA polymerase to transcribe a limited number of intermediate genes (*step 4*). DNA replication proceeds via a rolling hairpin mechanism (*steps 3 and 5*) similar to that for parvoviruses and is concurrent with the expression of the intermediate gene products (*step 4*). The intermediate gene products include transcription factors required for late gene expression (*step 6*). The products of late gene expression encode most of the structural proteins as well as the viral transcriptase and

associated factors that will be required at the start of a new infection. Progeny genomes, viral structural proteins, and membranes of the host cell participate in the assembly of viral particles that undergo extensive morphogenesis and maturation (*step 7*) prior to release.

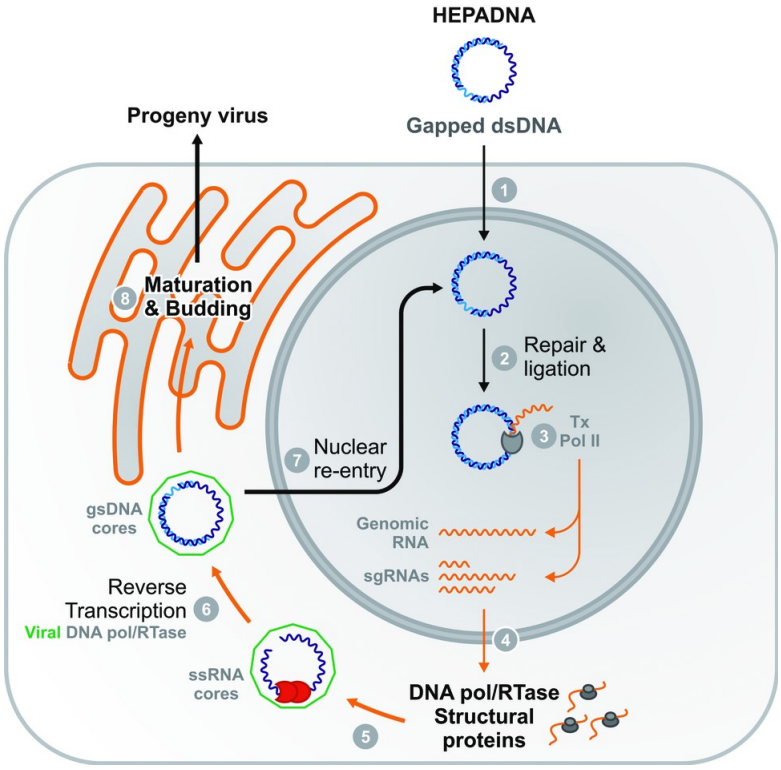

FIGURE 5.8 **Simplified replication scheme for *Hepadnaviridae***. Following entry and partial uncoating (*step 1*), viral cores containing the gapped dsDNA genome migrate to the nucleus where the dsDNA genome is repaired and ligated into a covalently closed circle (*steps 1 and 2*). This repaired genome is transcribed by the host-cell RNA polymerase II (*step 3*) to yield terminally redundant pregenomic RNA and subgenomic RNAs for the viral structural and nonstructural proteins (*step 4*). In the cytoplasm, the viral DNA polymerase, reverse transcriptase (RTase), and core proteins associate with the pregenomic RNA to form immature cores (*step 5*) that mediate polymerase-primed reverse transcription of the pregenomic RNA to yield gapped dsDNA genomes (*step 6*). The progeny cores either then enter the nucleus (*step 7*) to repeat the cycle or can bud through the endoplasmic reticulum to yield viral particles (*step 8*).

Because cellular DNA synthesis occurs during the S phase of the cell cycle and not at all in terminally differentiated G0 cells, viruses that depend on the host DNA polymerase must either wait for the infected cell to enter S phase spontaneously, as in the case of parvoviruses, or early in infection, they must express one or more viral oncogenes to override the regulation of the cell-cycle control proteins p53 or pRb and thereby stimulate infected cells to enter S phase, as in polyomaviruses and papillomaviruses. Inactivation of pRb releases cellular transcription factor E2F, which induces expression of the cellular DNA polymerase α primase, DNA polymerase δ, ssDNA binding protein, and several critical cellular enzymes that are involved in both the *de novo* and the salvage pathways of deoxynucleotide triphosphate (dNTP) biosynthesis, including ribonucleotide reductase, thymidylate synthetase, dihydrofolate reductase, deoxyuridine triphosphate nucleotidohydrolase (dUTPase), and thymidine and thymidylate kinases. Viruses with large DNA genomes (eg, herpesviruses and poxviruses) encode some of those enzymes themselves and can thus replicate in nondividing cells and other environments that would not normally support DNA replication, such as terminally differentiated cells of the nervous system (some herpesviruses) or even the cytoplasm (poxviruses). Although these viral genes are often dispensable for virus replication in actively dividing cells in culture, they can exert a profound influence on viral virulence in infected organisms and thus provide targets for chemotherapeutic intervention. For example, the thymidine kinase gene of some herpesviruses (but not the host enzyme) phosphorylates the prodrug acyclovir to generate a dNTP analog that terminates nascent strands during DNA synthesis.[34]

Regulation of Viral Gene Expression

During the early, prereplicative phase of the infectious cycle of a typical DNA virus, a subset of so-called immediate early viral genes is expressed to produce mostly catalytic quantities of nonstructural proteins required for DNA replication and host-cell manipulation. After DNA replication, a different set of genes is expressed (late genes) that direct the synthesis of stoichiometric amounts of the structural proteins required for viral assembly. Expression of the early genes is often concomitantly repressed. This early/late switch, which is a common feature of many DNA virus families, can be understood as an evolutionary adaptation that gives viruses an advantage in competing with the host cell for gene expression: early gene expression is modest, whereas later during the postreplicative stage of the infectious cycle, increased gene copy numbers enable the virus to dominate the protein synthesis capacity of the cell. In addition to the early and late genes, sets of intermediate genes have been identified in the poxviruses and herpesviruses, with each temporal class encoding factors that switch on the next in a transcriptional cascade (Figs. 5.6 and 5.7). For the viruses in these families, transcription factors for immediate early genes are expressed late in infection and assembled into progeny virions in preparation for the next infectious cycle.

At the start of infection, immediate early viral promoters face stiff competition from overwhelming numbers of cellular promoters. To successfully recruit RNA pol II and other components of the transcriptional machinery, many DNA viral genomes contain enhancers: *cis*-acting regulatory elements that facilitate the assembly of transcription complexes by binding multiple cellular transcription factors and other accessory proteins. A defining feature of enhancers, which were first identified in the genome of the polyomavirus simian vacuolating virus 40 (SV40), is that they exert their effects from either upstream or downstream of promoters and can act over distances of several kilobases.

Efficient transcription from late promoters usually requires one or more early gene products, as well as cellular transcription factors that may differ from those used by the early promoters. Dependence on specific cellular transcription factors can limit the expression of late genes to particular cells or tissues where the necessary factors are naturally expressed. For example, transcription of late papillomavirus genes requires a specific transcription factor that is expressed only in fully differentiated skin cells. As a result, the replication cycle is stalled after DNA replication (Fig. 5.3, step 4) until the cell differentiates.

Infection of cells with herpesviruses and poxviruses increases the rate of mRNA degradation.[19] In some herpesviruses, that is mediated by a protein component of the infecting virions called vhs (virion host shutoff). Although enhanced turnover is not specific for cellular mRNAs, some host cell mRNAs are not subject to the suppression and viral mRNAs are readily replenished by robust transcription of the viral genome so that the net result is the selective suppression of host protein synthesis.[1] In addition, the rapid turnover of viral mRNAs accelerates the transitions in the transcriptional cascade.

Mechanisms of DNA Replication and Transcription

Most DNA viruses produce functional viral mRNAs by usurping the transcriptional machinery of the cell (Fig. 5.1). This machinery includes RNA pol II, multiple transcription factors, poly(A) polymerase, guanylyltransferase, methyltransferases, and the pathway of mRNA export from the nucleus. Even viruses with unusual genome structures such as parvoviruses and hepadnaviruses use these cellular components because their genomes are rendered into dsDNA before transcription (Figs. 5.4 and 5.8). Only DNA viruses that replicate in the cytoplasm (pox-, irido-, and asfiviruses) use virus-specific enzymes for transcription and posttranscriptional modification of their mRNAs (Fig. 5.7). Because these enzymes are virion structural components, they can often be purified more readily than their cellular counterparts, and in the case of the vaccinia poxvirus and *Chlorella* virus, their reactions and structural properties have been well studied.[51,59,61] The majority of RNA viruses also replicate in the cytoplasm and employ virus-specific enzymes to synthesize and modify their mRNA.[25]

Viral DNA genomes replicate by at least five different mechanisms, which are summarized as follows (for more details, readers are referred to the chapters that describe each viral family).

1. The circular dsDNA genomes of polyomaviruses and papillomaviruses (Fig. 5.3) replicate bidirectionally from a single AT-rich origin via the RNA-primed synthesis of continuous leading strands and discontinuous lagging strands at both replication forks. Circularity of the genome aside, the reactions at the replication forks closely resemble how the host chromosome is replicated.[37,77]

2. In stark contrast, the linear dsDNA genome of adenoviruses (Fig. 5.5) is replicated by a protein-primed synthesis of only the leading strand, resulting in displacement of ssDNA from each end of the parental duplex. The termini of the displaced strands anneal via inverted terminal repeats, creating duplex panhandle structures that serve as secondary origins of replication. The primer (preterminal protein) is the product of an early gene, and a copy of this protein is covalently bound to the 5′ end of each of the daughter strands.[23,75]

3. The linear dsDNA of herpesvirus genomes is first circularized and then replicated from one or more internal origins, most likely by an RNA-primed mechanism that eventually produces dsDNA concatamers (Fig. 5.6). Progeny DNA can undergo isomerization by homologous recombination between internal and terminal repeated sequences, and unit length genomes are resolved from the concatamers during packaging into virions.[83]

4. Despite their different structures and sizes, poxvirus (Fig. 5.7) and parvovirus (Fig. 5.4) genomes replicate by similar mechanisms. The close-ended duplex poxvirus genome (or the closed-ended duplex intermediate in parvovirus replication) is nicked near its terminus, and the newly generated 3′ end serves to prime DNA synthesis using the complementary strand of the duplex as template. This initial self-priming event is reproduced by partially base-paired hairpin structures located at each end of the duplex genome, resulting in so-called “rolling hairpin” replication. For both poxviruses and parvoviruses, the product is a dsDNA concatamer from which unit length genomes are excised by resolution of concatamer junctions.[10,20,123]

5. Finally, in the most tortuous mechanism of all, hepadnaviruses (Fig. 5.8) replicate their dsDNA genome by a full-length pregenomic ssRNA transcript made by RNA polymerase II. Pregenomic RNA is then reverse transcribed by the viral-encoded DNA polymerase/reverse transcriptase to produce dsDNA progeny. In contrast to retroviruses (Fig. 5.9), DNA integration is not required for hepadnavirus replication, the genome being maintained as a circular episome in the nucleus of infected cells. Caulimoviruses—the only dsDNA viruses that infect plants—use a similar reverse transcriptase (RT)-mediated replication strategy. To prime first-strand DNA synthesis, hepadnavirus RTase uses a domain of the polymerase itself. This differs from the tRNA-primed strategy employed by retroviruses and caulimoviruses.

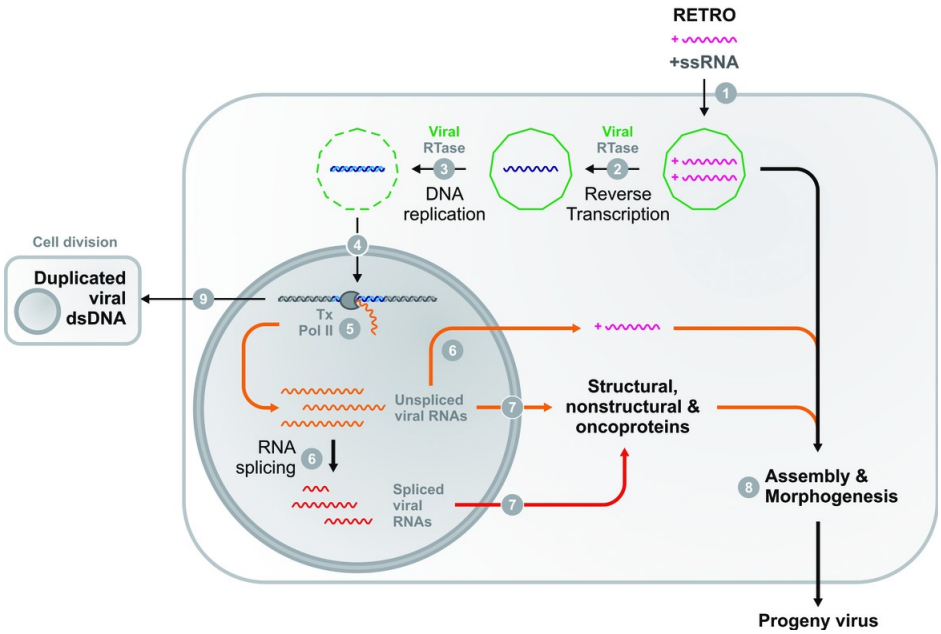

FIGURE 5.9 **Simplified replication scheme of *Retroviridae***. Following entry and partial uncoating (*step 1*), the viral genome is copied into dsDNA by the reverse transcriptase (*steps 2 and 3*) and integrated into the host chromosome by the virion DNA integrase (*step 4*). The integrated viral genome (provirus) is transcribed by the host-cell RNA polymerase II (*step 5*) to produce viral transcripts that function as precursors to the mRNA for the viral proteins (*steps 6 and 7*) as well as progeny genomes for assembly into infectious particles (*step 8*).

The polymerases employed for these strategies of replication are structurally and functionally homologous—yet they accomplish replication via very distinct mechanisms. Thus, the evolutionary origin of such disparate mechanisms of replication remains uncertain.

Remarkably, protein-primed replication is discontinuous. In the three known examples—adenovirus, poliovirus, and hepadnaviruses—the first few nucleotides of the genome are templated from an internal motif rather than at the very 3′ end of the parental genome. This necessitates a jump or realignment of the protein-primer product together with the polymerase to the 3′ end of the parental genome to complete synthesis of the daughter strand. In the case of adenovirus, the first templated nucleotides added to the primer are positions 4-6 of the genome, which then realigns with the 3′ end of the genome to complete daughter-strand synthesis following annealing of the nascent strand to the first three nucleotides of the parental genome.[23] For hepadnaviruses, the first four nucleotides are added to the RTase from a stem loop positioned at the 5′ end of the pregenomic RNA. The RTase nascent strand RNA product leaps almost 3 kb and then continues processively to complete synthesis of the daughter strand. Among the RNA viruses, the picornaviruses employ an internal stem loop termed the *cis*-acting replication element (cre) within the parental strand to template the uridylylation of a protein primer, VPg.[92] This primer is then repositioned together with polymerase at the 3′ end of the genome to prime synthesis of the new strand. Such protein-primed mechanisms likely aid in maintaining the integrity of the genome ends, which contain vital signals for replication.

Latent and Persistent Infections

In addition to the typical vegetative replication cycles illustrated in Figures 5.3 through 5.8, many DNA viruses establish latent or persistent infections of their hosts. Several distinct mechanisms of persistence have been identified with different viruses, but they all involve suppression of viral cytopathic effects, long-term maintenance of the viral genome, and evasion of the cellular and organismal defenses. For example, herpesviruses typically establish latent infections in which the viral genome is maintained as a circular episome in the nucleus, expressing at most only a few viral genes and yielding no infectious virus. Such latent herpesvirus infections persist throughout the life of the host, successfully evading host immune surveillance, yet able to reemerge at intervals as productive lytic infections. Because of its importance for human health, understanding the establishment and maintenance of herpesvirus latency and the mechanisms that regulate the reemergence of infectious virus are the subject of intense study (see Volume 2, Chapters 8-15). In the case of human herpesvirus 4 (Epstein-Barr virus), which infects B lymphocytes and causes mononucleosis, latent infections can be established and maintained in cell culture; this has greatly facilitated experimental study of the mechanisms involved. For other herpesviruses, the establishment and maintenance of latency occur in less-accessible cell types that pose additional challenges to understanding latency.

Viral Oncogenes and Neoplastic Transformation

In cells that somehow survive DNA virus infection, such as nonpermissive cells that express early genes but cannot replicate viral DNA or produce infectious progeny, the expression of viral oncogenes and the consequent loss of cell cycle control can lead to neoplastic transformation and the formation of tumors in infected animals. Unlike typical retrovirus-induced tumors, where the entire viral genome is integrated into the host chromosome as an essential step of the viral replication cycle, tumors induced by DNA viruses rarely contain a complete viral genome or produce infectious virus. Instead, they typically express only the viral oncogenes from integrated copies, disrupting cell cycle control by inactivating p53/pRb or by activating cyclin-dependent kinases, for example. Alone among the DNA viruses that replicate in the nucleus, parvoviruses do not induce tumors because they are unable to override cell cycle controls. Some poxviruses induce the formation of self-limiting benign tumors when they secrete a virus-encoded growth factor that induces surrounding cells to divide.

Expression and Replication of RNA Virus Genomes

RNA Virus Genome Strategies

The type of RNA genome dictates the first biosynthetic steps following infection. For example, the message sense positive-strand RNA viruses, excluding the retroviruses, all initially deliver their genomes to ribosomes to ensure the synthesis of essential proteins to establish viral replication (Figs. 5.10 and 5.11). Consequently, the viral genomic RNA alone is infectious once delivered into a host cell—a fact that greatly facilitated the genetic manipulation of such viruses. That the RNA alone was infectious was first shown for tobacco mosaic virus, in experiments that helped establish that genes were comprised of nucleic acids.[44] By contrast to the genomes of positive-sense RNA viruses, those of the negative-strand RNA viruses, retroviruses, and double-stranded RNA viruses all must deliver into the cell a protein RNA complex that contains within it the viral polymerase.

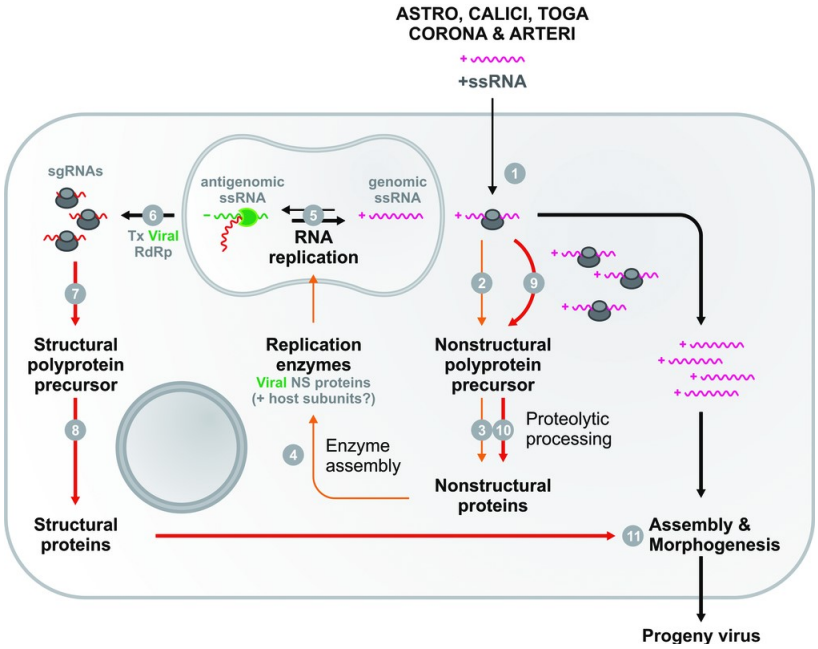

FIGURE 5.10 **Simplified replication scheme of positive-strand RNA viruses that produce subgenomic RNA**. Following entry and uncoating (*step 1*), the genomic RNA is engaged by the host-cell ribosome to produce the nonstructural proteins including the RdRp (*steps 2 and 3*). The viral replication enzymes together with host components form replication compartment in which the genomic RNA is replicated into an antigenome and progeny genomes (*steps 4 and 5*). The viral RdRp also transcribes one or more subgenomic RNAs (*step 6*) that encode viral structural proteins (*steps 7 and 8*). Replicated genomes are translated to amplify the production of viral proteins (*steps 9 and 10*) and may be used as templates for further replication. The genomes are assembled with viral structural proteins (*step 11*) to yield progeny virions. The scheme by which the *Coronaviridae* and *Arteriviridae* synthesize their subgenomic RNA is different from that employed by the *Toga-*, *Astra-*, and *Caliciviridae*.

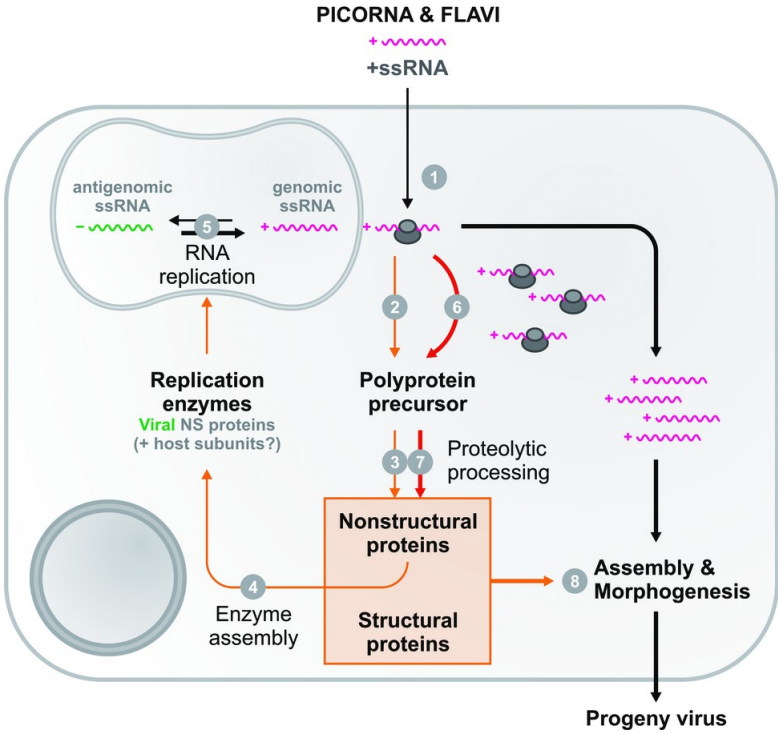

FIGURE 5.11 **Schematic of the replication cycle of positive-strand RNA viruses that do not make subgenomic RNA**. Following entry and uncoating (*step 1*), the genomic RNA is used directly as an mRNA to synthesize both structural and nonstructural proteins (*steps 2 and 3*). The nonstructural proteins, including the RdRp and host components, establish a membrane-associated replication compartment in which the genomic RNA is copied into antigenomic RNA and progeny genomes (*steps 4 and 5*). Replication produces additional genomic RNA that can be used for further translation and genomic amplification (*steps 6, 7, and 5*) as well as assembly with the viral structural proteins (*step 8*) to yield progeny virions.

The positive-sense RNA viruses fall into two general groups, those that transcribe subgenomic RNAs (Fig. 5.10) and those that do not (Fig. 5.11). Viruses that do not transcribe subgenomic RNA are translated by the host ribosomes to produce one or more polyprotein precursors that undergo a set of in *cis* and in *trans* cleavage reactions by viral-encoded proteases.[30] Those cleavage reactions result in the production of the individual nonstructural proteins essential for the replication of the viral genome, including the establishment of the site of viral replication. For viruses that produce a single polyprotein, the precursors of the catalytic components are generated in equimolar amounts to the structural proteins. In some cases, control over the abundance of the catalytic components is provided by the accumulation of intermediates in the processing cascade in which one or more of the catalytic activities are absent.[110] Such components can play crucial structural or regulatory roles in the establishment of replication compartments. Viruses that produce two polyprotein precursors always employ an internal ribosome entry site (IRES) to drive the expression of the second polyprotein. Because the translation of the second polyprotein is independent of the first, the proteins are not produced in equimolar amounts, allowing for some regulation of the abundance of the different proteins.[57] For viruses that produce subgenomic RNAs, the input genome is first translated into a precursor of the nonstructural proteins that includes the RdRp. The genome is then subsequently copied into one or more subgenomic mRNAs that encode the structural proteins. The production of subgenomic mRNAs facilitates the synthesis of distinct amounts of the structural and nonstructural proteins—such that the structural proteins are often produced in vast molar excess of the catalytically essential nonstructural proteins.

In contrast to the naked RNA of the positive-sense RNA viruses, that of the negative-sense, ambisense, and dsRNA viruses is noninfectious (Figs. 5.12-5.14). This is because the infectious unit is a ribonucleoprotein complex comprising the genomic RNA associated with the necessary viral polymerase components for synthesis of mRNA.[84,124] In the case of the negative-sense and ambisense RNA viruses, the input genomic RNA is copied by the viral polymerase complex into mRNA (Figs. 5.12 and 5.13). The input genomes are then replicated to yield antigenomes, a process that requires newly synthesized viral nucleocapsid protein to coat the nascent RNA strand.[84,124] For the negative-sense RNA viruses (Fig. 5.12), the antigenomes are positive sense, but they do not serve as templates for translation, rather they function exclusively as templates for genomic replication.[84,124] The negative-sense orthomyxoviruses and bornaviruses replicate within the host-cell nucleus, whereas the mammalian infecting rhabdoviruses as well as the paramyxoviruses and filoviruses replicate in the cytoplasm. A further important distinction is that the rhabdo-, filo-, paramyxo-, and bornaviruses sequentially transcribe a series of 5-10 monocistronic mRNAs from a single genomic template, whereas the orthomyxoviruses synthesize only a single mRNA from each segment. In the case of the ambisense RNA viruses, the genomic and antigenomic RNAs have both message and antimessage polarity. Those RNAs are not directly translated and instead serve as templates for synthesis of single monocistronic mRNAs during transcription or as templates for genome replication (Fig. 5.13).[36] In contrast to all other negative-strand RNA viruses, the circular ssRNA genome of HDV does not require a specialized viral polymerase for copying. Rather, the HDV genome is transcribed and replicated by the host DNA-dependent RNA polymerase II in a unique RNA-templated reaction.[112]

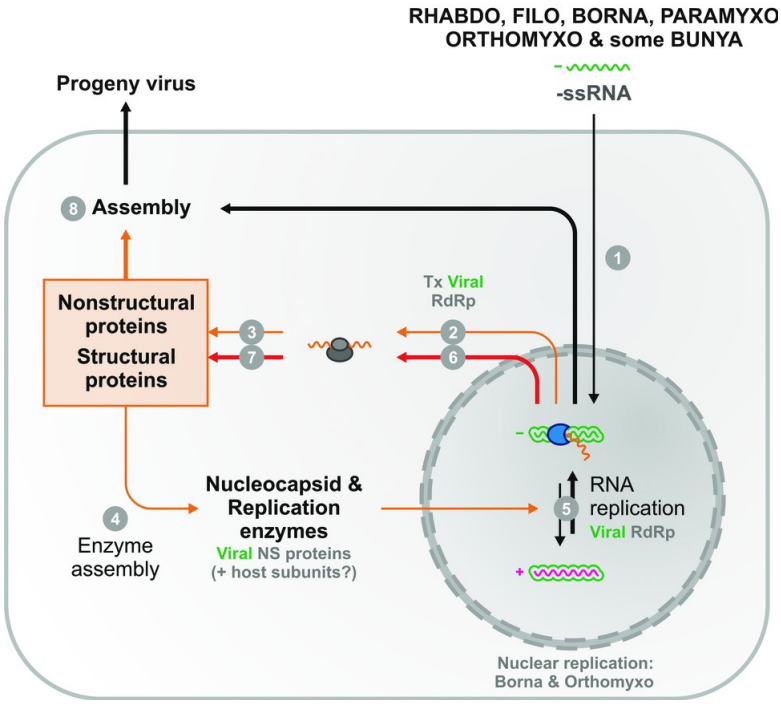

FIGURE 5.12 **Simplified replication scheme for negative-sense RNA viruses**. Following entry and partial uncoating (*step 1*), the encapsidated viral genomic RNAs are transcribed by the virion RdRp into mRNAs (*step 2*) that encode the viral structural and nonstructural proteins (*step 3*). For the *Orthomyxoviridae* and *Bornaviridae*, transcription occurs in the nucleus (reflecting their need for splicing), whereas for the *Rhabdo*, *Filo*, *Paramyxo*, and *Bunyaviridae*, this occurs in the cytoplasm. Replication is concomitant with encapsidation of the genomic and antigenomic RNAs (*steps 4 and 5*), and the newly produced genomic RNAs serve as templates for further mRNA production (*step 6*) as source of further viral proteins (*step 7*) as well as for assembly of progeny virions (*step 8*). The segmented negative-sense RNA viruses transcribe each genomic segment into a single transcript (which can be alternately spliced in the case of the orthomyxoviruses). The nonsegmented negative-sense RNA viruses sequentially transcribe a series of 5 to 10 monocistronic

mRNAs from the genomic template.

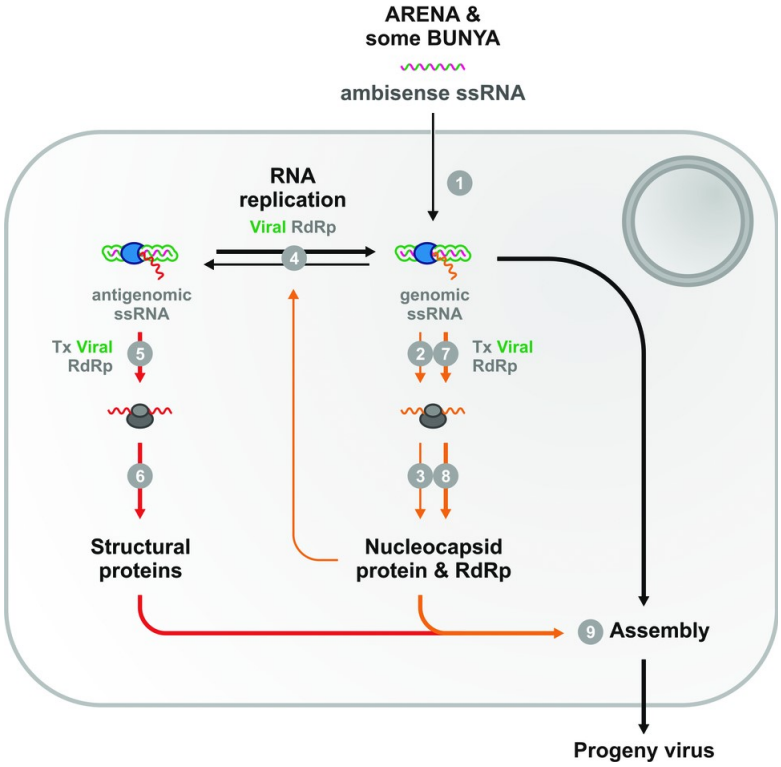

FIGURE 5.13 **Replication scheme for the ambisense RNA viruses**. Following entry and partial uncoating (*step 1*), the encapsidated genomic RNAs are transcribed by the virion RdRp to yield mRNAs (*step 2*) that encode the viral nucleocapsid protein and RdRp (*step 3*). These proteins catalyze the synthesis of antigenomic RNA (*step 4*), which serves both as templates for transcription of additional mRNAs (*step 5*) that encode the remaining viral proteins (*step 6*), as well as templates for production of more genomic RNA (*step 4*). The replication products (both genomic and antigenomic) can serve as further templates for transcription of mRNA that encode both viral structural and nonstructural proteins (*steps 5 through 8*). The genomic RNAs are also assembled together with the structural proteins into infectious viral particles (*step 9*). Note some bunyaviruses are also simply negative sense.

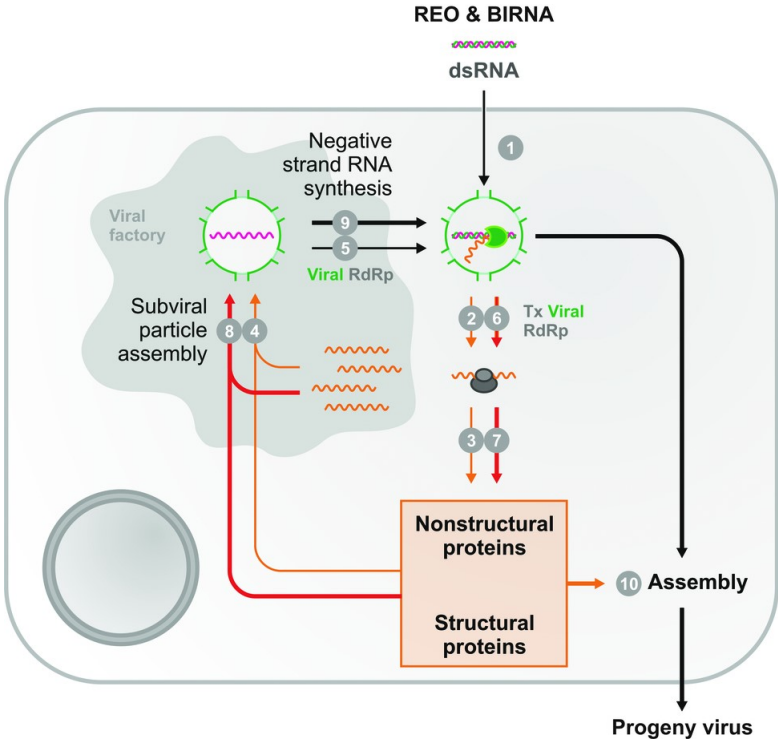

FIGURE 5.14 **Simplified replication scheme for dsRNA viruses**. Following entry and partial uncoating (*step 1*), the dsRNA segments within viral cores are transcribed by the core-associated RdRp to produce mRNA (*step 2*) for the viral proteins (*step 3*). These form subviral particles around the mRNA (*step 4*), which are then copied to produce genomic dsRNAs (*step 5*). RNA synthesis and replication likely occur within a specific cytoplasmic factory established by the viral proteins. Progeny subviral particles contribute to viral

gene expression (*steps 6 and 7*) and replication (*steps 8 and 9*) and assemble with outer shell proteins to form progeny virus particles (*step 10*).

For the dsRNA viruses, the segmented genomes are delivered into the cell as a subviral particle that remains intact for transcription of the mRNA (Fig. 5.14). The polymerase and RNA-modifying enzymes form a structural element through which the nascent mRNA strand passes and is cotranscriptionally modified. The ssRNA and their encoded proteins reassemble into new subviral particles that can direct the synthesis of antigenome RNA and form a dsRNA genome. Those progeny subviral particles can serve as templates for the production of further mRNA, and the viral protein or can be packaged into new virions.[78]

The positive-sense RNA genomes of retroviruses (Fig. 5.9) use a distinct mechanism for replication. Here, the incoming ssRNA is copied into a dsDNA provirus using the virally encoded reverse transcriptase—an essential component of the incoming virion.[6,114] This dsDNA is integrated into the host genome, where it is copied into differentially spliced transcripts that either serve as template for production of viral proteins or for production of viral progeny.

Regulation of Gene Expression

In contrast to DNA viruses, which exhibit clearly demarked early/late-phase gene expression, most RNA viruses show little differentiation between the pre- and postreplicative phases of the infectious cycle and express their genes at roughly the same relative levels throughout infection. In cases where some temporal regulation of gene expression occurs, the differences are subtle and mostly accompanied at the transcriptional level by modulation of mRNA levels.

Although DNA viruses utilize alternate translational control mechanisms as well as RNA viruses, translational control is especially prevalent among RNA viruses. The rate-limiting step for translation of cellular mRNA by the ribosome is at the process of initiation, and more specifically recognition of the mRNA cap structure by the cap-recognition protein eIF-4E, part of a multi-subunit complex. The cap-recognition complex then facilitates the recruitment of the small subunit of the ribosome in complex with other initiation factors. This complex then scans to the first AUG on the template RNA, typically localized within 100 or so nucleotides of the mRNA cap structure. Following this scanning to the AUG, the small subunit is joined by the large ribosomal subunit, and polypeptide chain synthesis is initiated. The poly(A) tail present at most cellular mRNA structures stimulates translation, as it is bound by the poly(A)-binding protein that bridges interactions with the eIF-4G component of the cap-recognition complex. This may serve to functionally circularize the mRNA to facilitate ongoing translation. There are many notable exceptions to this general mechanism that have been exploited by RNA viruses. This reflects the fact that although superficially many RNA virus positive-sense transcripts resemble cellular mRNAs, there are distinctions at both the 5′ and 3′ ends of the RNA that are accompanied frequently by an altered mechanism of translational initiation.

One of the best known examples of an altered mechanism of translational initiation was provided by studies of how the genomes of picornaviruses—which lack a 5′ mRNA cap structure and instead contain a genome-linked protein VPg—are expressed. The 5′ untranslated regions of picornavirus genomes are unusually long (several 100s of nucleotides, including several AUG-specifying triplet) and are highly structured. Those structured elements termed internal ribosome entry sites (or IRES) serve to recruit the ribosome directly to the viral RNA[93] without the need for a full complement of initiation factors for translation. Indeed, in some viruses, such as cricket paralysis virus, the ribosome is recruited to the IRES without the need for any of the initiation factors that are essential for conventional translation. Such altered mechanisms are exploited by viruses to facilitate the efficient translation of the viral mRNA, thereby outcompeting cellular transcripts for translation ensuring the robust synthesis of viral proteins.

There are also many RNA viruses that produce mRNAs that lack a polyadenylate tail, the dsRNA reoviruses and rotaviruses, many of the ambisense arenaviruses and bunyaviruses, as well as numerous positive-strand RNA viruses. On most cellular mRNAs, the polyadenylate tail is generally thought to function as an element that stabilizes the mRNA and additionally favors translation by facilitating recycling of ribosomes through a protein-mediated bridging mechanism that brings the 5′ and 3′ ends of the mRNA together. Such circularization is likely achieved by direct RNA-RNA interactions in the case of some positive-strand RNA plant viruses—such as the luteovirus, barley yellow dwarf virus.[65]

A further mechanism of regulation of translation initiation exploited by RNA viruses is a translation termination-reinitiation strategy. This strategy involves the termination of translation of an upstream open-reading frame followed by the reinitiation at a proximal (<40 nt away) downstream open-reading frame. The stop and start codons are frequently found in an overlapping arrangement, but in distinct reading frames to one another, and termination is essential for the subsequent reinitiation. This strategy is exploited by the positive-strand RNA calciviruses, such as feline calicivirus and murine norovirus, as well as the negative-strand RNA viruses influenza B virus and respiratory syncytial virus.[97] Evidence from studies of calicivirus translation implicates the presence of a secondary structure in the mRNA that may function to “tether” the small ribosomal subunit to the transcript before reengagement of the large subunit.

Suppression of termination is also exploited during the translation of many viral mRNAs, notably those of the alphavirus and retrovirus families, as a mechanism of producing a protein with an extended C-terminus. In the case of some retroviruses, a *cis*-acting regulatory element at the Gag-Pol junction functions to suppress termination of translation by leading to the misreading of the UAG termination codon to form the Gag-Pol polyprotein. Processing of this polyprotein then provides the viral reverse transcriptase and integrase. A second strategy is used by Rous sarcoma virus as well as other retroviruses, termed ribosomal frameshifting. A slippery sequence in the template leads to a realignment of the tRNA and the subsequent accessing of an altered reading frame. Such a mechanism is also exploited by other positive-strand viruses (notably the *Coronaviridae*) as well as several DNA viruses.

Structural and Nonstructural Proteins

By definition, virus-specified structural proteins are incorporated into virus particles, whereas nonstructural proteins are found only in infected cells. However, negative-sense, ambisense, and dsRNA viruses assemble their RdRps and associated enzymes into progeny virions and, therefore, encode predominantly or exclusively structural proteins. In addition to the polymerase, virus-encoded enzymes often include one or more proteases, an RNA helicase, mRNA capping enzymes, poly(A) polymerase, sometimes a nuclease, and in the case of retroviruses, a DNA integrase.

The proteases process the primary translation products of which they are a part by cleaving them at highly specific target sequences. Host

proteins are also subject to proteolysis by viral proteases conditioning the infected cell environment to favor viral replication. In some picornavirus-infected cells, inhibition of host-cell protein synthesis is facilitated by viral protease–mediated cleavage of the eIF-4G component of the cap-recognition complex. Similarly, protease-mediated cleavage of nuclear pore components blocks mRNP export. Particularly among the larger RNA viruses, RNA helicases may be required to disrupt intermolecular or intramolecular base pairing during RNA synthesis, although some RdRps are capable of melting RNA duplexes without help. Guanylyl and methyltransferases construct the 5′ caps found on the mRNAs of most eukaryotic RNA viruses (reviewed in Ref.[24]) except for the picornaviruses, which are uncapped, and the orthomyxoviruses, arenaviruses, and bunyaviruses, which acquire their caps by stealing them from the host using a virus-encoded cap-dependent endonuclease.[81,96] The cap structures of the *Mononegavirales* are formed by an altered mechanism involving a guanosine diphosphate (GDP)—polyribonucleotidyltransferase present within the same polypeptide chain that contains the polymerase and mRNA cap methylase activities.[72,73,85,86] A similar mechanism operates for the coronaviruses where a domain of the viral polymerase nsp12 transfers RNA to the amino terminus of the nsp9 protein and subsequently to GDP.[89] At their 3′ ends, most animal virus mRNAs carry untemplated poly(A) tails, although 3′ tRNA-like structures are common among plant RNA viruses. Polyadenylation is usually ascribed to a stuttering side reaction of the viral RdRp rather than to a separate poly(A) polymerase, as found in poxviruses, although the enzymology of the reaction remains to be clearly defined.[84,124] Comparisons among the amino acid sequences of viral RdRps establish clear phylogenetic relationships that also reflect other differences in viral genome structures and strategies. Moreover, the atomic structures of viral RdRps show clear resemblance to one another as well as to reverse transcriptases and DNA-dependent RNA and DNA polymerases. Although the overall levels of amino acid sequence homology among these diverse polymerases are statistically insignificant, RdRps share many of the characteristic polymerase motifs that occupy critical positions in the three-dimensional architecture of the polymerase active site. Taken together the structural similarities of their RdRps firmly anchor the RNA viruses to the rest of the biosphere and suggest that despite their diversity these viruses probably escaped from cellular origins to establish a horizontally transmissible parasitic existence using polymerases that radiated from a common ancestor.

Host Cell Factors

Proteins provided by the host undoubtedly play essential roles in RNA virus replication, although different cellular proteins have been implicated in different virus systems. The clearest example comes from the RNA replicase enzymes of the RNA bacteriophages Q*β* and MS2, which, in addition to the single phage-specified polypeptide that provides the polymerase active site, contain four cell-specified subunits: the *Escherichia coli* ribosomal protein S1, two translation elongation factors (EF-Tu and EF-Ts), and a strand-specific RNA-binding protein, host factor 1.[9] In view of the similarity of their host components, the distinct specificities of the Qβ and MS2 replicases for their cognate RNA templates must be determined by their unique viral subunits. The unexpected recruitment of translation factors to assist RNA replication —despite the evident differences between these two processes—may reflect underlying biochemical similarities in the RNA–protein interactions involved.

Translation factors have also been implicated in the replication of RNA viruses of eukaryotes. For example, a subunit of the initiation factor eIF-3 binds to the brome mosaic virus RdRp and increases its activity,[100] and several other host proteins have been found to associate with viral RNAs in infected cells. One of the difficulties in definitively assigning whether such proteins are just passengers, or active players in the replication of the viral genome, is often the lack of *in vitro* replication assays and/or genetically tractable host systems. Substantial progress has been made in this regard with the use of genetic, chemical, RNAi, as well as proteomic screens to hunt for host factors that are required for viral replication. Reconstructing the replication cycle of viruses in yeast[62,88] has permitted screens in collections of gene knockouts in *Saccharomyces cerevisiae*, and the application of RNAi has extended such screens to invertebrate and vertebrate cells.[17] The use of haploid human cells and gene-trap retroviruses allowed the discovery of host-cell factors that are essential for the entry into cells of a number of viral pathogens.[13,14] Most recently, the application of genome-wide loss of function screens based on CRISPR-mediated inactivation of host genes has led to an explosion of information on the virus-host relationship (reviewed in Ref.[98]). Although in some cases the overlap of “hits” between seemingly similar screens is low—likely reflecting technical difficulties of such large scale screens—the convergence on specific pathways exploited by viruses for their replication is generally high.[45] Such systematic analyses provide a potentially fertile source for understanding the virus-host relationship; however, the detailed mechanistic understanding of how specific host proteins mediate viral replication lags behind.

In addition to host proteins, host nucleic acids also appear to participate directly in the replication of some RNA viruses. One such example is provided by host micro RNAs that function to titrate cellular gene expression by promoting transcript turnover or by sequestration. Work with hepatitis C virus (HCV) identified a liver specific microRNA-122—which regulates cholesterol biosynthesis—that binds to two sites at the 5′ end of the viral genome and enhances HCV replication.[63] Additional host miRNA that regulate HCV replication both positively and negatively have been identified.[74] Such control of viral replication extends beyond the RNA viruses and further serves to underscore the intimate relationship between viruses and their host cells.

Compartmentalization of Replication Sites

Unlike the phage replicases, the RdRps of eukaryotic viruses are invariably found associated with some type of supramolecular assembly: host-cell membranes for positive-sense RNA viruses, nucleocapsids frequently found as inclusionlike structures for the negative-sense RNA viruses, and subviral particles arranged as factories for the dsRNA viruses. The positive-strand RNA viruses (Figs. 5.10 and 5.11) represent the best-characterized examples of membrane-associated replication complexes.[26] In most cases, the membranes are extensively rearranged, often—although not exclusively—derived from the endoplasmic reticulum, and invariable topologically arranged as an invagination of the membrane of the endoplasmic reticulum (ER) to form an open-necked crucible. Within each vesicle are a relatively small number of genomic templates —possibly as few as one, along with the viral replication machinery and cellular proteins that appear to play a role in the formation of the vesicular structure. In the case of the flaviviruses, genome wide loss of function screens identified the host oligosaccharyltransferase (OST) complex as required for viral RNA replication, playing a structural role in this process likely tethering the replication machinery to the membrane.[76] The prevailing model is that such vesicular structures represent the sites of viral RNA replication, and that the nascent RNA chains are rapidly exported to the cytoplasm. In many cases, virion assembly sites are juxtaposed to such compartmentalized replication sites, which likely facilitates the coordination of the replication and assembly of viral particles.

For the negative-sense RNA viruses that replicate in the cytoplasm (Fig. 5.12), the viral replication machinery is typically found in inclusionlike structures that are often rich in cellular chaperones such as heat shock protein (hsp)70. Although such inclusionlike structures are not essential for RNA synthesis—they are absent at the onset of infection and at a time at which the intracellular environment is likely most hostile for viral

replication—such structures have been shown to be active sites of RNA synthesis.[54,68] Likewise, dsRNA viruses establish factories or viroplasms (Fig. 5.13) in the infected cell that are sites of RNA synthesis and assembly.[11] Such compartmentalization of the replication machinery is, therefore, a conserved theme across a spectrum of viruses. Although such sequestration may both facilitate catalysis and sequester the viral replication machinery from surveillance by host defense machinery, it is largely uncertain what events lead to the establishment of such sites. Although there is a strong correlation between compartment formation and viral replication, examination of the very initial replication events including establishment of such sites remains challenging.

Mechanisms of RNA Replication and Transcription

The simplest mechanism of RNA replication is that used by HDV in which rolling circle synthesis by RNA polymerase II makes multimeric RNAs of both positive and negative polarity. *cis*-Acting ribozymes then cleave linear RNA monomers from these concatamers and covalently circularize them to produce mature antigenomes and genomes, respectively.[112] Although this simple mechanism is shared by several viroids and other subviral RNA pathogens of plants, HDV is the only RNA pathogen of vertebrates known to replicate by a rolling circle mechanism. Far more commonly, ssRNA genomes are replicated via the synthesis of complementary RNA monomers produced by successive rounds of strand displacement during end-to-end copying of linear templates.[12] However, both ends of the RNA are generally required for template activity, suggesting that even linear RNAs may be functionally circularized, perhaps to facilitate reiterative replication and hinder erosion of the termini.

The relative abundance of the genomic and antigenomic RNAs is regulated in different ways in different virus families. In the case of Qβ, negative-strand RNA synthesis is limited by the requirement for an additional host subunit that is not required for positive-strand RNA synthesis.[9] In other virus systems, distinct *cis*-acting RNA signals are largely responsible for determining the relative template activities of the complementary strands, although it is possible that different host factors are involved here as well. For the togavirus Sindbis, negative-sense RNA is synthesized only by a short-lived version of the RdRp, which is an intermediate in the proteolytic processing pathway of the viral nonstructural proteins.[64] As infection proceeds, increased viral protease activity cleaves this transient intermediate and thereby switches the template specificity of the RdRp to the synthesis of positive-strand RNA.

There are two general mechanisms of initiation by RdRps: primer-dependent and primer-independent mechanisms. The majority of RNA viruses appear to employ a primer-independent mechanism. Some of the best studied examples of this are provided by the bacteriophages Qβ, MS2, and ϕ6, as well as by eukaryotic viruses HCV, and reo-, rota-, vesicular stomatitis virus, and influenza A virus. The general mechanism by which *de novo* initiation occurs is that the RdRp initiates synthesis by forming a phosphodiester bond between the initiation nucleoside triphosphate (iNTP) and a second NTP. Atomic structures of polymerases engaged in the steps of initiation have provided remarkable insights into this process including the identification of residue that stabilize the initiating nucleotides termed “priming residues.” The second general mechanism is a primer-dependent mechanism of initiation. The primers themselves can come in different flavors, for example, the picornavirus RdRps use a small viral-encoded protein, VPg, that is covalently uridylylated by the polymerase on an internal RNA structure at a single A residue.[92] This primer, polymerase complex then relocalizes to the terminus to prime the initiation reaction. Nucleic acids can also serve as primers for RNA synthesis, and here there are two currently known variations of this mechanism of primed initiation. The first of these is exploited by the RdRps of the segmented negative-strand RNA viruses: *Orthomyxo-*, *Bunya-*, and *Arenaviridae* to prime messenger RNA synthesis. Here, a virally encoded endonuclease cleaves a short capped primer from either host-cell pre-mRNA in the case of the *Orthomyxoviridae* or mature cytoplasmic mRNA in the case of the *Arena-* and *Bunyaviridae*.[96] Such primers then base pair with the ends of the template to prime synthesis of the viral mRNA. Sequence analysis of the stolen host cell mRNA primers in influenza virus–infected cells identified that some primers contain a host-derived translation initiation AUG, which can yield additional proteins or N-terminal extensions on viral proteins.[58] Remarkably, these cap-snatching polymerases can also initiate “*de novo*.” For the arena and bunyaviruses, this *de novo* initiation occurs not by initiation of synthesis opposite the terminal nucleotide but by initiation at the penultimate nucleotide followed by a subsequent realignment of the nascent strand to “prime” the process of replication.[41] This strategy termed “prime and realign” may help ensure the integrity of the termini is maintained during copying. For the orthomyxovirus influenza A virus, the process of prime and realign is only employed during synthesis of the genomic RNA copy of the segments. During synthesis of the antigenomic copy, the polymerase initiates at the 3′ terminus directly.[87]

Summary and Perspectives

Mechanistic studies of viral replication have led us to some of the most fundamental discoveries in biology and provided critical knowledge to facilitate the development of vaccines and antiviral therapeutics to combat disease. The global burden of infectious disease caused by existing viruses and the ongoing threat of emerging viral infections further emphasizes the need to fully understand the mechanisms by which viruses replicate. Such studies promise to continue to uncover strategies to combat disease and to harness viral replication for beneficial purposes both in the laboratory as expression vectors, gene knockout tools, neural circuit tracers, and probes of cellular, organismal, and population biology but also in the clinic as potential vaccines, delivery vehicles, and imaging tools. The industrial applications of viruses are equally profound—as polymers, as well as tools to make microcircuits and even as potential fuel cells. All these applications of viruses have depended on our current understanding of the replication machinery and the strategies of viral replication. With the ability to simply manipulate viral genomes combined with the new burst in discovery of viruses from large scale sequencing projects and the ease with which host genomes can now be manipulated the future of fundamental studies of viral replication promises to be even more informative than the exciting past.

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REFERENCES

1. Abernathy E, Glaunsinger B. Emerging roles for RNA degradation in viral replication and antiviral defense. Virology. 2015;479-480:600-608.

2. Albertini AA, Ruigrok RW, Blondel D. Rabies virus transcription and replication. Adv Virus Res. 2011;79:1-22.

3. Albertini AA, Wernimont AK, Muziol T, et al. Crystal structure of the rabies virus nucleoprotein-RNA complex. Science.

2006;313(5785):360-363.

4. Arslan D, Legendre M, Seltzer V, Abergel C, Claverie JM. Distant Mimivirus relative with a larger genome highlights the fundamental features of Megaviridae. Proc Natl Acad Sci U S A. 2011;108(42):17486-17491.

5. Bahar MW, Graham SC, Chen RA, et al. How vaccinia virus has evolved to subvert the host immune response. J Struct Biol. 2011;175(2):127-134.

6. Baltimore D. RNA-dependent DNA polymerase in virions of RNA tumour viruses. Nature. 1970;226(5252):1209-1211.

7. Barr JN, Fearns R. How RNA viruses maintain their genome integrity. J Gen Virol. 2010;91(Pt 6):1373-1387.

8. Bernhardt HS. The RNA world hypothesis: the worst theory of the early evolution of life (except for all the others)(a). Biol Direct. 2012;7:23.

9. Blumenthal T, Carmichael GG. RNA replication: function and structure of Qbeta-replicase. Annu Rev Biochem. 1979;48:525-548.

10. Boyle KA, Stanitsa ES, Greseth MD, Lindgren JK, Traktman P. Evaluation of the role of the vaccinia virus uracil DNA glycosylase and A20 proteins as intrinsic components of the DNA polymerase holoenzyme. J Biol Chem. 2011;286(28):24702-24713.

11. Broering TJ, Kim J, Miller CL, et al. Reovirus nonstructural protein mu NS recruits viral core surface proteins and entering core particles to factory-like inclusions. J Virol. 2004;78(4):1882-1892.

12. Buck KW. Comparison of the replication of positive-stranded RNA viruses of plants and animals. Adv Virus Res. 1996;47:159-251.

13. Carette JE, Guimaraes CP, Varadarajan M, et al. Haploid genetic screens in human cells identify host factors used by pathogens. Science. 2009;326(5957):1231-1235.

14. Carette JE, Raaben M, Wong AC, et al. Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. Nature. 2011;477(7364):340-343.

15. Chakrabarti A, Jha BK, Silverman RH. New insights into the role of RNase L in innate immunity. J Interferon Cytokine Res. 2011;31(1):49-57.

16. Chang L, Godinez WJ, Kim IH, et al. Herpesviral replication compartments move and coalesce at nuclear speckles to enhance export of viral late mRNA. Proc Natl Acad Sci U S A. 2011;108(21):E136-E144.

17. Cherry S, Doukas T, Armknecht S, et al. Genome-wide RNAi screen reveals a specific sensitivity of IRES-containing RNA viruses to host translation inhibition. Genes Dev. 2005;19(4):445-452.

18. Claverie JM, Abergel C, Ogata H. Mimivirus. Curr Top Microbiol Immunol. 2009;328:89-121.

19. Clyde K, Glaunsinger BA. Getting the message direct manipulation of host mRNA accumulation during gammaherpesvirus lytic infection. Adv Virus Res. 2010;78:1-42.

20. Cotmore SF, Tattersall P. High-mobility group 1/2 proteins are essential for initiating rolling-circle-type DNA replication at a parvovirus hairpin origin. J Virol. 1998;72(11):8477-8484.

21. Crotty S, Cameron CE, Andino R. RNA virus error catastrophe: direct molecular test by using ribavirin. Proc Natl Acad Sci U S A. 2001;98(12):6895-6900.

22. Daffis S, Szretter KJ, Schriewer J, et al. 2′-O methylation of the viral mRNA cap evades host restriction by IFIT family members. Nature. 2010;468(7322):452-456.

23. de Jong RN, van der Vliet PC, Brenkman AB. Adenovirus DNA replication: protein priming, jumping back and the role of the DNA binding protein DBP. Curr Top Microbiol Immunol. 2003;272:187-211.

24. Decroly E, Canard B. Biochemical principles and inhibitors to interfere with viral capping pathways. Curr Opin Virol. 2017;24:87-96.

25. Decroly E, Ferron F, Lescar J, Canard B. Conventional and unconventional mechanisms for capping viral mRNA. Nat Rev Microbiol. 2011;10(1):51-65.

26. den Boon JA, Ahlquist P. Organelle-like membrane compartmentalization of positive-strand RNA virus replication factories. Annu Rev Microbiol. 2010;64:241-256.

27. Deng T, Vreede FT, Brownlee GG. Different de novo initiation strategies are used by influenza virus RNA polymerase on its cRNA and viral RNA promoters during viral RNA replication. J Virol. 2006;80(5):2337-2348.

28. Denison MR, Graham RL, Donaldson EF, Eckerle LD, Baric RS. Coronaviruses: an RNA proofreading machine regulates replication fidelity and diversity. RNA Biol. 2011;8(2):270-279.

29. Domingo E, Holland JJ. RNA virus mutations and fitness for survival. Annu Rev Microbiol. 1997;51:151-178.

30. Dougherty WG, Semler BL. Expression of virus-encoded proteinases: functional and structural similarities with cellular enzymes. Microbiol Rev. 1993;57(4):781-822.

31. Dreher TW. Role of tRNA-like structures in controlling plant virus replication. Virus Res. 2009;139(2):217-229.

32. Eckerle LD, Lu X, Sperry SM, Choi L, Denison MR. High fidelity of murine hepatitis virus replication is decreased in nsp14 exoribonuclease

mutants. J Virol. 2007;81(22):12135-12144.

33. Eigen M. Viral quasispecies. Sci Am. 1993;269(1):42-49.

34. Elion GB, Furman PA, Fyfe JA, de Miranda P, Beauchamp L, Schaeffer HJ. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl) guanine. Proc Natl Acad Sci U S A. 1977;74(12):5716-5720.

35. Emerman M, Malik HS. Paleovirology—modern consequences of ancient viruses. PLoS Biol. 2010;8(2):e1000301.

36. Emonet SE, Urata S, de la Torre JC. Arenavirus reverse genetics: new approaches for the investigation of arenavirus biology and development of antiviral strategies. Virology. 2011;411(2):416-425.

37. Fanning E, Zhao K. SV40 DNA replication: from the A gene to a nanomachine. Virology. 2009;384(2):352-359.

38. Favoreel HW, Van de Walle GR, Nauwynck HJ, Pensaert MB. Virus complement evasion strategies. J Gen Virol. 2003;84(Pt 1):1-15.

39. Ganser-Pornillos BK, Pornillos O. Restriction of HIV-1 and other retroviruses by TRIM5. Nat Rev Microbiol. 2019;17(9):546-556.

40. Gao Q, Park MS, Palese P. Expression of transgenes from newcastle disease virus with a segmented genome. J Virol. 2008;82(6):2692-2698.

41. Garcin D, Kolakofsky D. Tacaribe arenavirus RNA synthesis in vitro is primer dependent and suggests an unusual model for the initiation of genome replication. J Virol. 1992;66(3):1370-1376.

42. Genoyer E, Lopez CB. The impact of defective viruses on infection and immunity. Annu Rev Virol. 2019;6(1):547-566.

43. Gerlier D, Lyles DS. Interplay between innate immunity and negative-strand RNA viruses: towards a rational model. Microbiol Mol Biol Rev. 2011;75(3):468-490, second page of table of contents.

44. Gierer A, Schramm G. Infectivity of ribonucleic acid from tobacco mosaic virus. Nature. 1956;177(4511):702-703.

45. Goff SP. Knockdown screens to knockout HIV-1. Cell. 2008;135(3):417-420.

46. Goodbourn S, Randall RE. The regulation of type I interferon production by paramyxoviruses. J Interferon Cytokine Res. 2009;29(9):539-547.

47. Green TJ, Zhang X, Wertz GW, Luo M. Structure of the vesicular stomatitis virus nucleoprotein-RNA complex. Science. 2006;313(5785):357-360.

48. Gribble J, Stevens LJ, Agostini ML, et al. The coronavirus proofreading exoribonuclease mediates extensive viral recombination. PLoS Pathog. 2021;17(1):e1009226.

49. Guo LT, Adams RL, Wan H, et al. Sequencing and structure probing of long RNAs using marathonRT: a next-generation reverse transcriptase. J Mol Biol. 2020;432(10):3338-3352.

50. Guogas LM, Filman DJ, Hogle JM, Gehrke L. Cofolding organizes alfalfa mosaic virus RNA and coat protein for replication. Science. 2004;306(5704):2108-2111.

51. Håkansson K, Doherty AJ, Shuman S, Wigley DB. X-ray crystallography reveals a large conformational change during guanyl transfer by mRNA capping enzymes. Cell. 1997;89(4):545-553.

52. Haller O, Weber F. Pathogenic viruses: smart manipulators of the interferon system. Curr Top Microbiol Immunol. 2007;316:315-334.

53. Hay S, Kannourakis G. A time to kill: viral manipulation of the cell death program. J Gen Virol. 2002;83(Pt 7):1547-1564.

54. Heinrich BS, Cureton DK, Rahmeh AA, Whelan SP. Protein expression redirects vesicular stomatitis virus RNA synthesis to cytoplasmic inclusions. PLoS Pathog. 2010;6(6):e1000958.

55. Heinrich BS, Maliga Z, Stein DA, Hyman AA, Whelan SPJ. Phase transitions drive the formation of vesicular stomatitis virus replication compartments. mBio. 2018;9(5).

56. Herold J, Andino R. Poliovirus RNA replication requires genome circularization through a protein-protein bridge. Mol Cell. 2001;7(3):581-591.

57. Hertz MI, Thompson SR. Mechanism of translation initiation by Dicistroviridae IGR IRESs. Virology. 2011;411(2):355-361.

58. Ho JSY, Angel M, Ma Y, et al. Hybrid gene origination creates human-virus chimeric proteins during infection. Cell. 2020;181(7):1502-1517 e23.

59. Hodel AE, Gershon PD, Quiocho FA. Structural basis for sequence-nonspecific recognition of 5′-capped mRNA by a cap-modifying enzyme. Mol Cell. 1998;1(3):443-447.

60. Holland JJ, De La Torre JC, Steinhauer DA. RNA virus populations as quasispecies. Curr Top Microbiol Immunol. 1992;176:1-20.

61. Hu G, Gershon PD, Hodel AE, Quiocho FA. mRNA cap recognition: dominant role of enhanced stacking interactions between methylated bases and protein aromatic side chains. Proc Natl Acad Sci U S A. 1999;96(13):7149-7154.

62. Janda M, Ahlquist P. RNA-dependent replication, transcription, and persistence of brome mosaic virus RNA replicons in *S. cerevisiae*. Cell. 1993;72(6):961-970.

63. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science. 2005;309(5740):1577-1581.

64. Kim KH, Rümenapf T, Strauss EG, Strauss JH. Regulation of Semliki Forest virus RNA replication: a model for the control of alphavirus pathogenesis in invertebrate hosts. Virology. 2004;323(1):153-163.

65. Kneller EL, Rakotondrafara AM, Miller WA. Cap-independent translation of plant viral RNAs. Virus Res. 2006;119(1):63-75.

66. Kranzusch PJ, Schenk AD, Rahmeh AA, et al. Assembly of a functional Machupo virus polymerase complex. Proc Natl Acad Sci U S A. 2010;107(46):20069-20074.

67. Kranzusch PJ, Whelan SP. Arenavirus Z protein controls viral RNA synthesis by locking a polymerase-promoter complex. Proc Natl Acad Sci U S A. 2011;108(49):19743-19748.

68. Lahaye X, Vidy A, Pomier C, et al. Functional characterization of Negri bodies (NBs) in rabies virus-infected cells: evidence that NBs are sites of viral transcription and replication. J Virol. 2009;83(16):7948-7958.

69. Lauring AS, Andino R. Quasispecies theory and the behavior of RNA viruses. PLoS Pathog. 2010;6(7):e1001005.

70. Lee HR, Kim MH, Lee JS, Liang C, Jung JU. Viral interferon regulatory factors. J Interferon Cytokine Res. 2009;29(9):621-627.

71. Lee YF, Nomoto A, Detjen BM, Wimmer E. A protein covalently linked to poliovirus genome RNA. Proc Natl Acad Sci U S A. 1977;74(1):59-63.

72. Li J, Fontaine-Rodriguez EC, Whelan SP. Amino acid residues within conserved domain VI of the vesicular stomatitis virus large polymerase protein essential for mRNA cap methyltransferase activity. J Virol. 2005;79(21):13373-13384.

73. Li J, Rahmeh A, Morelli M, Whelan SP. A conserved motif in region v of the large polymerase proteins of nonsegmented negative-sense RNA viruses that is essential for mRNA capping. J Virol. 2008;82(2):775-784.

74. Li Q, Lowey B, Sodroski C, et al. Cellular microRNA networks regulate host dependency of hepatitis C virus infection. Nat Commun. 2017;8(1):1789.

75. Liu H, Naismith JH, Hay RT. Adenovirus DNA replication. Curr Top Microbiol Immunol. 2003;272:131-164.

76. Marceau CD, Puschnik AS, Majzoub K, et al. Genetic dissection of Flaviviridae host factors through genome-scale CRISPR screens. Nature. 2016;535(7610):159-163.

77. McBride AA. Replication and partitioning of papillomavirus genomes. Adv Virus Res. 2008;72:155-205.

78. McDonald SM, Patton JT. Assortment and packaging of the segmented rotavirus genome. Trends Microbiol. 2011;19(3):136-144.

79. McFadden G, Mohamed MR, Rahman MM, Bartee E. Cytokine determinants of viral tropism. Nat Rev Immunol. 2009;9(9):645-655.

80. Mercer J, Schelhaas M, Helenius A. Virus entry by endocytosis. Annu Rev Biochem. 2010;79:803-833.

81. Morin B, Rahmeh AA, Whelan SP. Mechanism of RNA synthesis initiation by the vesicular stomatitis virus polymerase. EMBO J. 2012;31(5):1320-1329.

82. Moya A, Elena SF, Bracho A, Miralles R, Barrio E. The evolution of RNA viruses: A population genetics view. Proc Natl Acad Sci U S A. 2000;97(13):6967-6973.

83. Muylaert I, Tang KW, Elias P. Replication and recombination of herpes simplex virus DNA. J Biol Chem. 2011;286(18):15619-15624.

84. Neumann G, Brownlee GG, Fodor E, Kawaoka Y. Orthomyxovirus replication, transcription, and polyadenylation. Curr Top Microbiol Immunol. 2004;283:121-143.

85. Ogino T, Banerjee AK. Unconventional mechanism of mRNA capping by the RNA-dependent RNA polymerase of vesicular stomatitis virus. Mol Cell. 2007;25(1):85-97.

86. Ogino T, Kobayashi M, Iwama M, Mizumoto K. Sendai virus RNA-dependent RNA polymerase L protein catalyzes cap methylation of virus-specific mRNA. J Biol Chem. 2005;280(6):4429-4435.

87. Oymans J, Te Velthuis AJW. A mechanism for priming and realignment during influenza A virus replication. J Virol. 2018;92(3):e01773-17.

88. Panavas T, Serviene E, Brasher J, Nagy PD. Yeast genome-wide screen reveals dissimilar sets of host genes affecting replication of RNA viruses. Proc Natl Acad Sci U S A. 2005;102(20):7326-7331.

89. Park GJ, Osinski A, Hernandez G, et al. The mechanism of RNA capping by SARS-CoV-2. Nature. 2022;609(7928):793-800.

90. Patel MR, Emerman M, Malik HS. Paleovirology—ghosts and gifts of viruses past. Curr Opin Virol. 2011;1(4):304-309.

91. Patton JT, Vasquez-Del Carpio R, Tortorici MA, Taraporewala ZF. Coupling of rotavirus genome replication and capsid assembly. Adv Virus Res. 2007;69:167-201.

92. Paul AV, Rieder E, Kim DW, van Boom JH, Wimmer E. Identification of an RNA hairpin in poliovirus RNA that serves as the primary template in the in vitro uridylylation of VPg. J Virol. 2000;74(22):10359-10370.

93. Pelletier J, Sonenberg N. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature. 1988;334(6180):320-325.

94. Pfeiffer JK, Kirkegaard K. Bottleneck-mediated quasispecies restriction during spread of an RNA virus from inoculation site to brain. Proc Natl Acad Sci U S A. 2006;103(14):5520-5525.

95. Philippe N, Legendre M, Doutre G, et al. Pandoraviruses: amoeba viruses with genomes up to 2.5 Mb reaching that of parasitic eukaryotes. Science. 2013;341(6143):281-286.

96. Plotch SJ, Bouloy M, Ulmanen I, Krug RM. A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell. 1981;23(3):847-858.

97. Powell ML. Translational termination-reinitiation in RNA viruses. Biochem Soc Trans. 2010;38(6):1558-1564.

98. Puschnik AS, Majzoub K, Ooi YS, Carette JE. A CRISPR toolbox to study virus-host interactions. Nat Rev Microbiol. 2017;15(6):351-364.

99. Pyle JD, Whelan SPJ, Bloyet LM. Structure and function of negative-strand RNA virus polymerase complexes. Enzymes. 2021;50:21-78.

100. Quadt R, Kao CC, Browning KS, Hershberger RP, Ahlquist P. Characterization of a host protein associated with brome mosaic virus RNA-dependent RNA polymerase. Proc Natl Acad Sci U S A. 1993;90(4):1498-1502.

101. Quinlan MP, Chen LB, Knipe DM. The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell. 1984;36(4):857-868.

102. Quiñones-Mateu ME, Arts EJ. Virus fitness: concept, quantification, and application to HIV population dynamics. Curr Top Microbiol Immunol. 2006;299:83-140.

103. Randow F, Lehner PJ. Viral avoidance and exploitation of the ubiquitin system. Nat Cell Biol. 2009;11(5):527-534.

104. Reich E, Franklin RM, Shatkin AJ, Tatum EL. Effect of actinomycin D on cellular nucleic acid synthesis and virus production. Science. 1961;134(3478):556-557.

105. Sawicki SG, Sawicki DL, Siddell SG. A contemporary view of coronavirus transcription. J Virol. 2007;81(1):20-29.

106. Schoggins JW. Interferon-stimulated genes: what do they all do? Annu Rev Virol. 2019;6(1):567-584.

107. Schütz S, Sarnow P. Interaction of viruses with the mammalian RNA interference pathway. Virology. 2006;344(1):151-157.

108. Sen GC, Sarkar SN. The interferon-stimulated genes: targets of direct signaling by interferons, double-stranded RNA, and viruses. Curr Top Microbiol Immunol. 2007;316:233-250.

109. Simon AE, Roossinck MJ, Havelda Z. Plant virus satellite and defective interfering RNAs: new paradigms for a new century. Annu Rev Phytopathol. 2004;42:415-437.

110. Strauss JH, Strauss EG. The alphaviruses: gene expression, replication, and evolution. Microbiol Rev. 1994;58(3):491-562.

111. Takeda M, Nakatsu Y, Ohno S, et al. Generation of measles virus with a segmented RNA genome. J Virol. 2006;80(9):4242-4248.

112. Taylor JM. Chapter 3. Replication of the hepatitis delta virus RNA genome. Adv Virus Res. 2009;74:103-121.

113. Taylor JM. Hepatitis D virus replication. Cold Spring Harb Perspect Med. 2015;5(11):a021568.

114. Temin HM, Mizutani S. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature. 1970;226(5252):1211-1213.

115. Tenthorey JL, Emerman M, Malik HS. Evolutionary landscapes of host-virus arms races. Annu Rev Immunol. 2022;40:271-294.

116. Thomas SM, Lamb RA, Paterson RG. Two mRNAs that differ by two nontemplated nucleotides encode the amino coterminal proteins P and V of the paramyxovirus SV5. Cell. 1988;54(6):891-902.

117. Versteeg GA, García-Sastre A. Viral tricks to grid-lock the type I interferon system. Curr Opin Microbiol. 2010;13(4):508-516.

118. Vignuzzi M, Stone JK, Arnold JJ, Cameron CE, Andino R. Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. Nature. 2006;439(7074):344-348.

119. Villordo SM, Gamarnik AV. Genome cyclization as strategy for flavivirus RNA replication. Virus Res. 2009;139(2):230-239.

120. Volchkov VE, Becker S, Volchkova VA, et al. GP mRNA of Ebola virus is edited by the Ebola virus polymerase and by T7 and vaccinia virus polymerases. Virology. 1995;214(2):421-430.

121. Wandzik JM, Kouba T, Cusack S. Structure and function of influenza polymerase. Cold Spring Harb Perspect Med. 2021;11(9):a038372.

122. Watts JM, Dang KK, Gorelick RJ, et al. Architecture and secondary structure of an entire HIV-1 RNA genome. Nature. 2009;460(7256):711-716.

123. Weitzman MD, Lilley CE, Chaurushiya MS. Genomes in conflict: maintaining genome integrity during virus infection. Annu Rev Microbiol. 2010;64:61-81.

124. Whelan SP, Barr JN, Wertz GW. Transcription and replication of nonsegmented negative-strand RNA viruses. Curr Top Microbiol

Immunol. 2004;283:61-119.

125. Wu Q, Wang X, Ding SW. Viral suppressors of RNA-based viral immunity: host targets. Cell Host Microbe. 2010;8(1):12-15.

126. Xu WK, Byun H, Dudley JP. The role of APOBECs in viral replication. Microorganisms. 2020;8(12):1899.

127. Zaccomer B, Haenni AL, Macaya G. The remarkable variety of plant RNA virus genomes. J Gen Virol. 1995;76 (Pt 2):231-247.

128. Zerbini FM, Siddell SG, Mushegian AR, et al. Differentiating between viruses and virus species by writing their names correctly. Arch Virol. 2022;167(4):1231-1234.

CHAPTER 6

Virus Assembly and Maturation

Christopher J. Neufeldt • Eric Hunter

Partitioning of proteins within the cell

Nuclear import and export of proteins and nucleic acids

The secretory pathway of the cell

Intracellular targeting and assembly of virion components

Assembly of nonenveloped viruses in the nucleus

Assembly of enveloped viruses in the nucleus

Assembly of viruses in the cytoplasm

Assembly of enveloped viruses at cellular membranes

Complex interactions with the secretory pathway

Modification of the secretory pathway

Incorporation of the nucleic acid genome during the assembly process

DNA viruses

RNA viruses

Postassembly modifications and virus release

Proteolytic cleavage and virus maturation

Nonproteolytic maturation events

Role of viral and cellular proteins in membrane extrusion

Mechanisms to facilitate the release of nascent particles

Cell-associated and noncanonical spread of infection

Virus assembly, a key step in the replication cycle of any virus, involves a process in which chemically distinct macromolecules are transported, often through different pathways, to a point within the cell where they are assembled into a nascent viral particle. A diversity of strategies and intracellular assembly sites are employed by members of the various virus families to ensure the efficient production of fully infectious virions. Nevertheless, a virus, irrespective of its molecular structure (membrane enveloped or nonenveloped) or the symmetry with which it assembles (icosahedral, spherical, or helical), must be able to take advantage of the intracellular transport pathways that exist within the cell if it is to achieve this goal. The end product of this selection process is assembly of each virus at a defined point within the cell. This chapter will focus on the cell biology of these intracellular targeting events, the intermolecular interactions that mediate targeting, and the assembly steps themselves.

Most viruses encode a very limited number of gene products. They are, therefore, dependent upon the cell not only for biosynthesis of the macromolecules that constitute the virus particle but also for the preexisting intracellular organelles and sorting mechanisms that the virus can utilize and, in some cases, modify to achieve delivery of those macromolecules to the sites of virion assembly. Since delivery depends on the same sorting mechanisms that the cell uses to delineate its subcellular organelles, the viral macromolecules must possess targeting signals similar to those of the components of those organelles. For a virus such as adenovirus, which assembles its nonenveloped capsids in the nucleus, this means that, following translation in the cytoplasm, each of the structural proteins of the mature virus must have the necessary protein-targeting information to be efficiently routed through the nuclear membrane to the assembly site. The situation is more complicated for a membrane-enveloped virus, such as influenza virus. For this virus, which assembles and releases virions from the apical surface of the epithelial cells that it infects, there is a necessity to ensure that the surface glycoproteins of the virus are correctly sorted by the secretory pathway of the cell to apical membranes. In addition, nucleocapsids, assembled in the nucleus, must be transported into and through the cytoplasm to the same location. As will be discussed below, the intracellular site at which the final phase of assembly and budding of an enveloped virus takes place is most often defined by the accumulation of the viral glycoproteins at a specific point in the secretory pathway of the cell. This also implies that there is specific molecular recognition of the virally encoded, membrane-spanning envelope components by the cytoplasmic nucleocapsids in order for a productive budding process to occur. Thus, interactions between proteins of viral and cellular origin, between viral proteins and nucleic acids and lipids, as well as between the viral proteins themselves are at the heart of the assembly process.

Partitioning of Proteins Within the Cell

For an actively growing eukaryotic cell, there is a constant need to transport proteins and nucleic acids from their site of synthesis to the specific intracellular domains where they must function (Fig. 6.1). Proteins synthesized on cytosolic ribosomes, for example, must be transported to specific regions of the cytoplasm, to mitochondria, and into the nucleus, while those synthesized on membrane-bound ribosomes will enter the

secretory pathway and be targeted to specific organelles along the way. At the same time, messenger RNAs (mRNAs) and integral RNA components of the ribosome, rRNAs, must be transported out of the nucleus. Proteins that participate in these intracellular trafficking processes have evolved to contain specific motifs that ensure the correct localization of the protein within the cell. Viruses have similarly evolved to take advantage of these preexisting pathways in order to accumulate, in specific locations, the necessary components for assembly of a nascent virus.

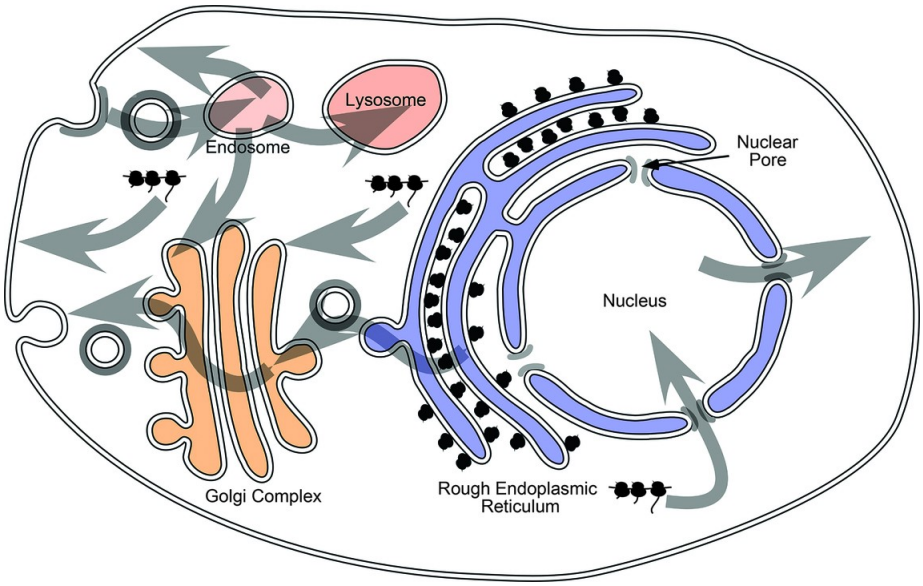

FIGURE 6.1 **Protein localization in a mammalian cell**. Membrane proteins destined for the plasma membrane traverse the secretory pathway of the cell. They associate with the endoplasmic reticulum cotranslationally and are translocated across the ER membrane via a proteinaceous pore—the translocon. Proteins are transported from the ER to the Golgi complex and on to the plasma membrane unless they contain specific amino acid motifs that localize or retain them at an intermediate location. Transport from one compartment to the next is via coated vesicles. Some membrane-spanning (integral membrane) proteins contain endocytosis motifs in their cytoplasmic domain that facilitate incorporation into clathrin-coated endocytic vesicles. Such proteins can be sorted back to the plasma membrane, to the trans-Golgi compartment of the secretory pathway, or to a lysosome for degradation. Proteins destined for the nucleus contain nuclear localization signals. These are short amino acid sequences that allow interaction with the nuclear pore machinery for transport across the nuclear membrane. Some proteins shuttle between the nucleus and cytoplasm and contain in addition a nuclear export signal.

Nuclear Import and Export of Proteins and Nucleic Acids

The Nuclear Pore Complex

Since the *nucleus* is segregated from the cytoplasm by an inner and outer membrane, access to and egress from this subcellular domain is mediated by specialized structures termed nuclear pore complexes (NPCs).[197,256] There are ~3000 NPCs on the nuclear envelope of an animal cell and each provides a proteinaceous channel between the nucleus and cytosol. The NPC itself is a very large structure with a molecular mass exceeding 50 mDa that exhibits eightfold symmetry and is constructed of multiple copies of ~30 different proteins called nucleoporins (Nups). Negative stain, combined with crystallography and cryoelectron microscope reconstructions of these complexes reveal a 125-nm diameter core structure in which eight spokes in a radially symmetrical arrangement join to form three main rings surrounding a central channel. Initial studies of partially purified NPCs indicated that this had a diameter of ~43 nm,[5,126,134] but recent cryoelectron tomography studies have revealed a substantially greater diameter—57-64 nm.[240,299] Attached to both faces of the central framework are peripheral structures, cytoplasmic filaments, and a nuclear basket assembly, which interact with molecules that transit the NPC.[78] The channel is filled with flexible, filamentous FG-nucleoporins, which are characterized by regions of multiple Phe-Gly repeats, and that form a “virtual gate” restricting transport into and out of the nucleus.[239] Small molecules and proteins may be able to passively diffuse through the NPC, but it acts as a molecular sieve for macromolecules. Larger proteins and macromolecular assemblages, such as the HIV capsid, must be actively moved through what is clearly a dynamic, malleable transporter structure that has the capacity to accommodate macromolecular complexes with diameters >55 nm.[299]

Nuclear Localization Signals

Proteins that are actively transported into or out of the nucleus are characterized by the presence of amino acid motifs that allow them to interact with the nuclear transport machinery. For import into the nucleus, these motifs are termed nuclear localization signals (NLS), and for export, nuclear export signals (NES). NLS motifs, such as that first identified in the SV40 T antigen,[120] are not only necessary for nuclear localization of the proteins in which they are present but are also sufficient to actively direct large foreign proteins, such as β-galactosidase, into the nucleus. Although there is no conservation of sequence in different NLS motifs, they are generally short (<20 amino acids), rich in basic amino acids, and frequently preceded by proline residues (Fig. 6.2A). Some NLS, such as that in the adenovirus DNA-binding protein, are bipartite and require two separate short clusters of basic residues in order to be functional.[298]

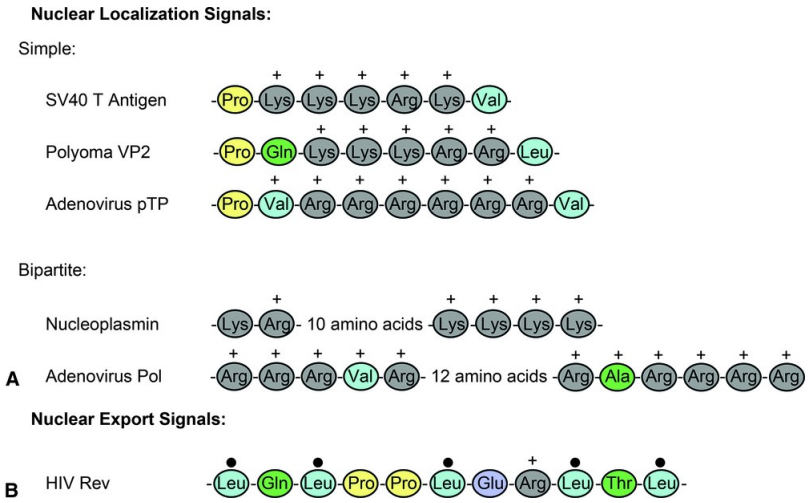

FIGURE 6.2 **Nuclear localization and export signals**. **A**. Nuclear localization signals (NLS) are the amino acid motifs that direct proteins into the nucleus are termed nuclear localization signals (NLS). Simple NLS sequences, such as those present in the SV40 virus T antigen or polyoma virus VP2, often contain a proline residue followed by a stretch of basic residues. This short sequence can relocate large proteins such as β-galactosidase into the nucleus. Bipartite NLS sequences, such as those present in nucleoplasmin or the adenovirus polymerase, are characterized by two stretches of basic amino acids separated by a variable spacer sequence. These NLS sequences are recognized by import receptor molecules such as importin-α. **B**. Proteins that shuttle into and out of the nucleus, such as Rev, possess a second motif, the nuclear export signal (NES). These motifs are characterized by a pattern of conserved leucine residues.

To be exported from the nucleus, proteins contain an NES. This nuclear transport signal is also short (~10 amino acids) and contains a pattern of conserved leucines[174] (Fig. 6.2B). Some proteins, such as Rev of HIV-1, possess both an NLS and an NES and appear to shuttle back and forth between the cytoplasm and the nucleus.[161]

Nuclear Transport Pathways—In and Out

The process of nuclear import is a two-stage process. In the first stage, the newly synthesized NLS-containing protein interacts with cytosolic receptor proteins that then bind to FG-Nups that make up the filaments on the cytoplasmic side of the NPC; a process referred to as “docking.” This complex is then translocated, in an energy-independent process, through the nuclear pore into the nucleus, where the complex is disassembled, allowing the transported protein to become functional.

The best-characterized protein import receptor is importin-α or karyopherin-α. After binding its NLS-containing cargo, importin-α interacts with importin-β, which then mediates docking with the NPC.[125] Most nuclear transport receptors (NTRs) belong to one large family of proteins (karyopherins), all of which share homology with importin-β (also named karyopherin-β). Members of this family have been classified as importins or exportins on the basis of the direction they carry their cargo. Importins and exportins are regulated by the small GTPase Ran, which is highly enriched in the nucleus in its GTP-bound form. Importins recognize their substrates in the cytoplasm and transport them through nuclear pores into the nucleus. In the nucleoplasm, RanGTP binds to importins, inducing the release of their cargoes. In contrast, exportins interact with their substrates only in the nucleus in the presence of RanGTP and release them after GTP hydrolysis in the cytoplasm, causing disassembly of the export complex (Fig. 6.3).[257] Thus, the directionality of transport is regulated by whether Ran is complexed with GDP or GTP.

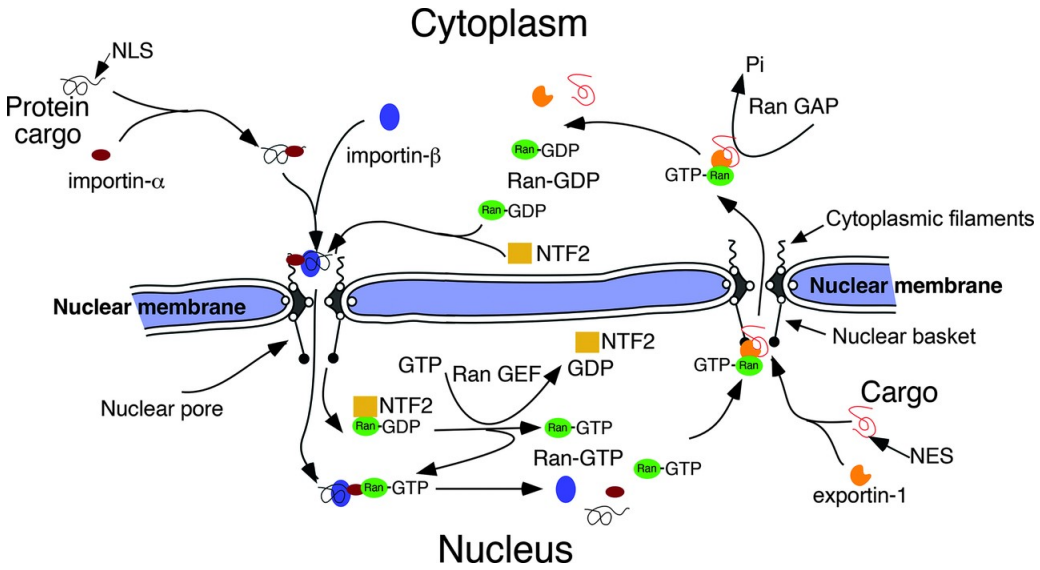

FIGURE 6.3 **Nuclear import and export pathways**. A protein bearing an NLS is recognized and bound by importin-α. Importin-β binds to this complex and carries it to the cytoplasmic filaments of the nuclear pore where together they mediate translocation of the

protein complex into the nucleus in an energy-independent process. In the nucleus, Ran-GTP binds to the importin complex and it dissociates delivering the protein cargo into the nucleus. Proteins destined for export out of the nucleus bind to exportin-1 via their NES. This complex together with Ran-GTP binds to nucleoporins localized to the nuclear basket of the pore complex and initiates translocation into the cytoplasm. Once there, Ran-GTP is converted to Ran-GDP by a Ran-GTPase–activating protein (RanGAP-1), causing the cargo-exportin-1-Ran complex to dissociate, thereby delivering the cargo to the cytoplasm.

Active transport of large molecules in either direction across the nuclear pore involves interaction with the FG-Nups. The FG repeats in these filamentous proteins provide binding sites for the nuclear transport receptors as well as other molecules, such as NTF2, involved in this process. While the exact mechanism by which the importin-cargo complex is carried through the nuclear pore is not known, recent studies have shown that low affinity interactions between NTRs and FG-Nup proteins allow a series of ultrafast docking and release cycles, which enable a rapid exchange of FG-motifs from the NTR binding pockets. This process is crucial for the fast and selective transport of NTR-cargo complexes through the NPC.[163,212]

The Secretory Pathway of the Cell

Proteins of both viral and cellular origin that are destined for the outer membrane of the cell travel along a highly conserved route known as the secretory pathway. This complex series of membrane-bound subcellular compartments, through which proteins pass sequentially, includes the endoplasmic reticulum (ER), an intermediate membrane compartment, and the cis-, medial-, and trans-compartments of the Golgi apparatus (Fig. 6.1). Proteins that traverse this pathway, such as the envelope glycoproteins of viruses, enter via the ER. Insertion of proteins into the ER occurs during translation, through a process termed translocation. The ER network of tubules and sacs defines a unique environment in which protein modification and folding can occur isolated from the cytoplasm. Because polyribosomes, in the process of translating proteins that are translocating into the secretory pathway, are bound tightly to the ER membrane, regions containing them are known as the rough ER.

Translocation

Translating ribosomes are directed to the ER membrane by a short sequence in the nascent polypeptide known as the signal peptide (SP). In most proteins, this 15-30 amino acid sequence, which contains a core of hydrophobic amino acids, is located at the N-terminus of the protein. Shortly after the SP emerges from the ribosome, it is bound by a ribonucleoprotein complex known as the signal recognition particle (SRP). SRP consists of a 300-nt 7SL RNA in complex with six protein subunits, RP9, SRP14, SRP19, SRP54, SRP68, and SRP72. SRP54 is a key component in cotranslational protein targeting, having a hydrophobic groove in which the SP is thought to bind. The act of SRP binding transiently arrests any further translation and directs the ribosome to an SRP-receptor on the ER (Fig. 6.4). The cycle of SP recognition by SRP, targeting to the SRP receptor and recycling of SRP into the cytoplasm, is regulated by GTP binding and hydrolysis. The latter is carried out by GTPases in SRP54 and the α-subunit of the SRP-receptor.[98,188]

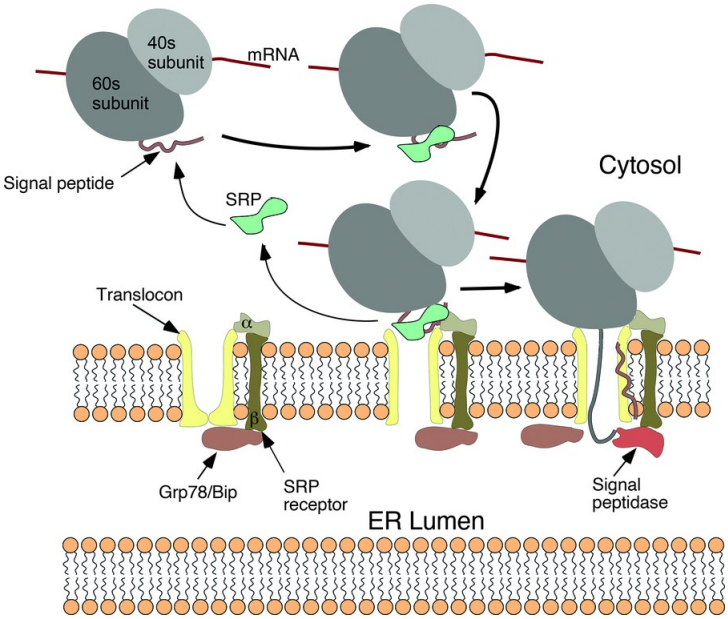

FIGURE 6.4 **Protein translocation into the secretory pathway**. Translation of a protein destined for the secretory pathway proceeds until the signal peptide exits the ribosome. The SRP binds to the signal peptide and the ribosome and arrests translation. The ribosome-SRP complex moves to the ER membrane where SRP binds to its receptor (SRP receptor). This interaction, with concomitant hydrolysis of bound GTP, releases SRP and mediates a tight interaction between the ribosome and a proteinaceous channel, the translocon or p61p. The release of SRP and binding of the signal peptide to components of the translocon induces a conformational change that widens a constriction in the channel and allows resumption of translation to occur. The chaperone protein, Grp78/Bip, present in the lumen, is poised to facilitate the folding of the nascent polypeptide chain as it emerges from the pore. The signal peptidase complex removes the signal peptide cotranslationally from those proteins that have a cleavable signal peptide. Secreted proteins will continue to traverse the translocon until they are completely located in the lumen of the ER. In contrast, for integral membrane proteins, translocation will stop following introduction of the hydrophobic anchor domain into the translocon, and transition into the lipid bilayer occurs through a single lateral opening in the pore.

Following the initial docking of the translationally arrested complex, the ribosome becomes tightly associated with the ER membrane via the translocon, a gated, aqueous, protein channel that spans the ER membrane. Concomitant with this process, the SRP and its receptor are released, the signal peptide is introduced into the channel, and translation resumes. The components and structure of the translocon have been

identified through biochemical and cryo-EM approaches in mammalian cells and genetic approaches in yeast. It is comprised of a heterotrimeric, multiple (12) membrane-spanning complex known as the Sec61p (composed of Sec61 alpha, beta, and gamma chains), which, based on crystal structures of archaebacterial Sec61p homologue SecYβγ, form a porelike central cavity with a single lateral opening to allow transition of membrane-spanning domains into the lipid bilayer.[272] Cryo-EM studies allowed the determination of the structure of a porcine 80S ribosome-Sec61 complex in both an idle and translating state at 3.4 and 3.9 Å resolution. This structure showed that Sec61 interacts with the ribosome primarily through conserved loops between helices 6 and 7 and between helices 8 and 9 of the α-subunit, as well as the N-terminal helix of Sec61γ.[276] Further high-resolution cryo-EM studies determined the structure of an active Sec61 channel that had been opened by a signal sequence, showing that the signal peptide could displace helix 2 of Sec61α allowing the former to move laterally toward the lipid bilayer (depicted graphically in Fig. 6.4).[277] Additional proteins (eg, the translocating chain-association membrane or TRAM protein) are necessary for optimal translocation. It is unlikely that the central pore of the translocon ever allows free diffusion between the cytosol and the lumen of the ER, since these compartments are chemically distinct. A constriction in the channel appears to be plugged by a short helix and widening of the constriction as well as displacement of the plug have been linked to conformational changes induced by SRP binding to the complex.[272] GRP78/Bip, a member of the Hsp70 family of chaperone proteins located in the lumen of the ER, plays multiple roles in gating the channel and facilitating translocation. The chaperone is then poised to facilitate the folding of the nascent polypeptide chain as it emerges from the pore, although this binding is not essential for translocation to proceed. The signal peptide, in those proteins with a transient N-terminal sequence, is cleaved from the rest of the polypeptide by a complex of five proteins called the signal peptidase shortly after it enters the lumen of the ER (Fig. 6.4).

For secreted proteins, translocation of the polypeptide through the translocon continues until the entire protein is present in the lumen of the ER. In contrast, integral membrane proteins, such as the envelope glycoproteins of viruses, contain a stop-transfer, membrane-anchor sequence that is generally located toward the C-terminus of the protein. Following the translation of this short (~25 amino acids) mostly hydrophobic sequence, the translocon undergoes a conformational change, likely similar to that observed with the signal peptide, which allows the membrane-spanning domain to be associated directly with the lipid bilayer. The product of this process is a type-I integral membrane protein (Fig. 6.5), such as the influenza virus hemagglutinin, in which the N-terminal ectodomain is in the lumen of the ER and the C-terminus is in the cytoplasm. In some proteins, such as the influenza virus neuraminidase, a longer N-terminal signal peptide also functions as the membrane anchor. In this case, the signal peptide is not cleaved from the polypeptide chain and the sequences C-terminal to it are translocated into the ER lumen, resulting in a type-II orientation. Multiple membrane-spanning proteins, such as the M-protein of the coronaviruses, appear to possess hydrophobic sequences that are alternatively recognized as signal and stop-transfer sequences, and translocation of such proteins may involve multiple Sec61 heterotrimers.[188,248]

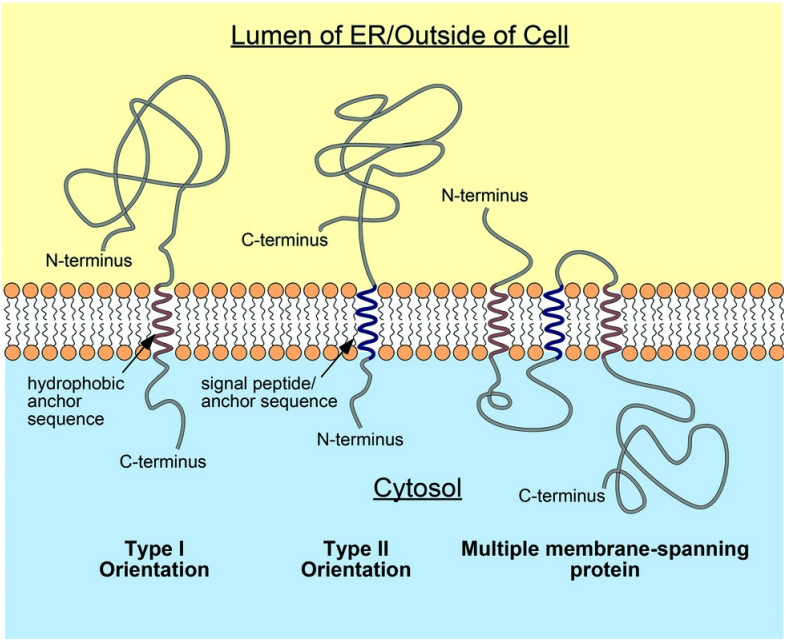

FIGURE 6.5 **Protein topology**. Proteins with type-I orientation generally have a cleavable N-terminal signal peptide that is removed cotranslationally from the nascent protein. The protein continues to be transferred into the lumen of the ER until a hydrophobic anchor sequence is translated and enters the translocon. Translocation then stops and the protein transitions from the protein pore into the lipid bilayer. Thus, type-I integral membrane proteins, such as the hemagglutinin of influenza virus, have their C-terminus in the cytoplasm and their N-terminus in the lumen of the ER (topologically equivalent to outside the cell). In type-II proteins, such as the influenza virus neuraminidase, the signal peptide forms the membrane anchor domain, so that at the end of translation, the C-terminal sequences are translocated into the lumen of the ER, leaving the N-terminus in the cytoplasm. For multiple membrane-spanning proteins, such as the M-protein of the coronaviruses, translocation is initiated at the first signal peptide sequence and continues until the first anchor domain. It is reinitiated following translation of a subsequent signal sequence and stopped again following translation of a second anchor. The exact mechanism by which this is accomplished without dismantling the translocon at intermediate steps in the process is not understood.

Posttranslational Modifications

Protein folding and quality control: Proteins enter the lumen of the ER in an unfolded state, and the process of folding into a transport-competent conformation is facilitated by interactions with molecular chaperones and folding enzymes located there. This collection of proteins includes BiP, calnexin (Cnx), calreticulin (Crt), GRP94, and protein disulfide isomerase (PDI). In addition to assisting in folding the nascent molecules, these proteins retain incompletely folded molecules in the lumen and act as a quality control system for the secretory pathway.[29]

Oligomeric proteins, such as the receptor/fusion proteins of enveloped viruses, also assemble into their quaternary conformation in this compartment. For many of these molecules, oligomerization appears to be a pre-requisite for transport out of the ER.

Glycosylation: A majority of proteins that traverse the secretory pathway are modified by the addition of oligosaccharide side chains either to the amino group of asparagines (N-linked glycosylation) or through the hydroxyl group of serines or threonines (O-linked glycosylation). N-Linked moieties are added cotranslationally in the lumen of the ER, where mannose-rich oligosaccharides are transferred by oligosaccharyltransferase from a lipid (dolichol) carrier to asparagine residues present in NXS/T motifs (where X is any amino acid but proline) within the protein. Trimming of terminal glucose and mannose residues from the branched oligosaccharide occurs in the ER and is closely linked with the Cnx- and Crt-mediated quality control process.[29] Further trimming of mannose residues followed by addition of other sugars (*N*-acetylglucosamine, galactose, fucose, and sialic acid), to yield complex oligosaccharide structures, occurs in the Golgi complex. O-Linked oligosaccharides are also added in this organelle.

Transport Through the Secretory Pathway

Transport of soluble and membrane-spanning proteins from one compartment of the secretory pathway to the next has traditionally been interpreted as being mediated by the formation of coated membrane vesicles that travel to and fuse with the target organelle.[13] Thus, once the process of protein folding and quality control has been completed in the ER, proteins would be sequestered into these transport vesicles prior to transit to the Golgi complex. While vesicular trafficking may occur for some proteins, recent studies suggest a more complex process involving specific regions of the ER known as the ER Exit Sites (ERES).[7,281] High-resolution microscopy studies show that these sites are composed of a cluster of vesicular-tubular membranes devoid of ribosomes near the ER.[281] Formation of these structures requires the coat protein II complex (COPII) that both stimulates membrane extrusion and plays a critical role in cargo protein selection.[56,281] Budding is initiated when a small myristylated protein (Sar1) is converted to the GTP-bound form, allowing it to bind to the membrane and recruit coat proteins (Sec23, Sec24, Sec13, and Sec31) in a stoichiometric manner.[56] Sorting signals displayed on the cytosolic surfaces of transmembrane protein cargo direct it into ERES, in some instances through physical association of the sec23-sec24 components in a sorting signal dependent manner.[128] Soluble proteins and transmembrane proteins lacking COPII sorting signals depend on a diversity of transmembrane adaptor proteins that can link them to the budding machinery. These include the ER-Golgi intermediate compartment-53 (ERGIC-53), p24 family of receptor proteins, as well as a set of multiple membrane-spanning ER vesicle (Erv14p, 26p, and 29p) proteins that can facilitate concentration of soluble proteins in COPII vesicles.[56]

Formation of the coat itself induces membrane curvature and vesicle budding. While initially thought to coat the vesicles bound for the Golgi complex, it now appears that COPII forms a collar that defines the boundary between the ER and the ERES, where it facilitates selective cargo concentration.[246] In mammalian cells, long “pearled” tubules, having the appearance of a series of interconnected vesicles, have been observed extending several microns from the ERES toward the Golgi complex and in close association with microtubules.[281] These transport tubules were associated with a second coat complex, COPI,[281] previously thought to only mediate retrograde transport of vesicles from the Golgi to the ER, as well as anterograde transport through the Golgi.[192]

A key question is how membrane traffic is directed to specific organelles within the cell, and over the past few decades, it has become clear that phosphoinositides are key determinants of compartmental membrane identity. As such they can direct membrane dynamics by providing spatial and temporal cues. Reversible phosphorylation and dephosphorylation of the inositol ring by phosphoinositide kinases and phosphatases generates seven distinct phosphoinositide species, which are specifically enriched in different organelles.[64] The plasma membrane is highly enriched in phosphatidylinositol 4,5-bisphosphate, while the Golgi apparatus and the *trans*-Golgi network (TGN) are characterized by phosphatidylinositol 4-phosphate. In contrast, endosomal vesicles are enriched in phosphatidylinositol 3-phosphate. Each specific membrane environment directly impacts the localization and activity of effector proteins and membrane-integral proteins such as receptors that interact with these lipids.[209] Such membrane receptors, incorporated into transport vesicles, can mediate docking of the vesicle with the target organelle and appear to define the specificity with which the cargo protein is delivered. Rab-GTPases and tethering proteins also appear to play an important role in defining the initial vesicle-target interactions. The latter are composed of two classes of protein: coiled-coil tethers (CCTs) and multisubunit tethering complexes.[21,292] SNAREs (soluble *N*-ethylmaleimide–sensitive factor attachment protein receptors) are generally accepted to mediate the final stage of vesicle docking and the subsequent membrane fusion events that are critical to transport.[23,103] A vesicle-specific SNARE (v-SNARE) interacts with a target-membrane–specific SNARE (t-SNARE) complex (generally comprised of three peptides) during this process. Two additional proteins, the *N*-ethylmaleimide–sensitive factor (Nsf) and soluble Nsf attachment proteins (SNAPs), act to disassemble the complex following fusion, allowing the SNARE components to be recycled.[103,110]

Protein Localization

Subcellular localization of proteins within the secretory pathway appears to be determined by a combination of sorting/targeting signals that mediate interactions with the coat complex for inclusion in a transport vesicle, and retention signals that localize the protein to a specific compartment within the secretory pathway. Localization is enhanced by the interplay of anterograde and retrograde transport that allows retrieval of proteins inadvertently transported beyond their target location. The classical example of this is the KDEL peptide sequence found on soluble proteins that are localized to the lumen of the ER.[171] Proteins containing this sequence are efficiently retrieved from the cis-Golgi by the KDEL receptor, which is incorporated into COPI vesicles for trafficking back to the ER. Similarly, membrane-spanning proteins localized to the ER have, at the C-terminus of the cytoplasmic domain, a dilysine (KKXX) COPI binding motif, which ensures their efficient retrieval from the Golgi complex.[108] This type of motif is utilized by the primate foamy viruses to concentrate the envelope glycoprotein complex (gp80/gp48) in the ER/intermediate compartment, where virus budding occurs.[87] For integral membrane proteins, retention signals often appear to be associated with the membrane-spanning domain(s) of the protein, as is the case for the coronavirus M protein, and may reflect preferred association with specific lipid compositions of the membrane within a particular component of the secretory pathway (see below).

The Golgi Complex

The Golgi complex represents a unique organelle within the secretory pathway in that it is comprised of a series of membrane bound compartments that are the sites for specific biochemical modifications to proteins and oligosaccharides, as well as locations where specific protein sorting decisions are made. Proteins transported from the ER enter the Golgi complex via the endoplasmic reticulum Golgi intermediate

compartment (ERGIC), and after traversing the cis-, medial-, and trans-cisternae, exit via the trans-Golgi network (TGN). The ERGIC matures in successive steps into cis-, medial-, and trans-Golgi and then the TGN through a process that is counterbalanced by retrograde transport of lipids and contents to the ER or earlier Golgi cisternae and is mediated by COPI-coated vesicles.[88,96] Each of the compartments provides a spatially distinct site for maintaining an ordered set of enzymes involved in the process of oligosaccharide maturation. They are also the sites at which proteins undergo O-linked glycosylation, through the addition, at certain serines and threonines, of monomeric sugar residues. It is in the trans-Golgi cisternae and trans-Golgi network where viral glycoprotein precursors, such as the Env polyprotein of the retroviruses, are cleaved to their mature forms through the action of members of the furin family of proteinases—enzymes that normally function to process cellular substrates such as polypeptide hormone precursors. This cleavage event is critical for the generation of a biologically functional glycoprotein and thus for virus infectivity.

Intracellular Targeting and Assembly of Virion Components

Viruses can be nominally divided into two groups based on the presence or absence of a lipid bilayer envelope. The nonenveloped viruses can assemble in the cytoplasm or nucleus and generally, for those that propagate in animal cells, exhibit icosahedral symmetry (see Chapter 3). For these viruses, the viral structural proteins and genomic nucleic acid must be targeted to or retained at the subcellular domain at which assembly occurs. Enveloped viruses, by their very nature, must acquire a lipid bilayer from one of the cell's membranes during the process of assembly. In some viruses, such as the herpesviruses and some retroviruses, this envelopment step takes place after the assembly of an intact capsid shell, while for others, the processes of envelopment and capsid assembly occur concomitantly. Some viruses undergo transient envelopment and in some cases re-envelopment during the process of assembly.

For nonenveloped viruses, the tightly assembled structure of the icosahedral shell forms a protective coat that prevents degradation of the genome by environmental factors. For enveloped viruses, the integrity of the nucleocapsid structure is less critical since the membrane provides a barrier to external degradative enzymes.

Assembly of Nonenveloped Viruses in the Nucleus

Many nonenveloped viruses, such as adenovirus, assemble their capsids in the nucleus. Adenoviruses are nonenveloped icosahedral viruses, 70-100 nm in diameter, that have a protein shell surrounding a DNA core. The protein shell (capsid) is composed of 252 capsomeres of which 240 are hexons and 12 are pentons. Each penton consists of a five-subunit base (polypeptide III) and a trimeric fiber (polypeptide IV) that extends out away from the shell. The hexon capsomeres are comprised of trimers of three tightly associated molecules of polypeptide II (Fig. 6.6).

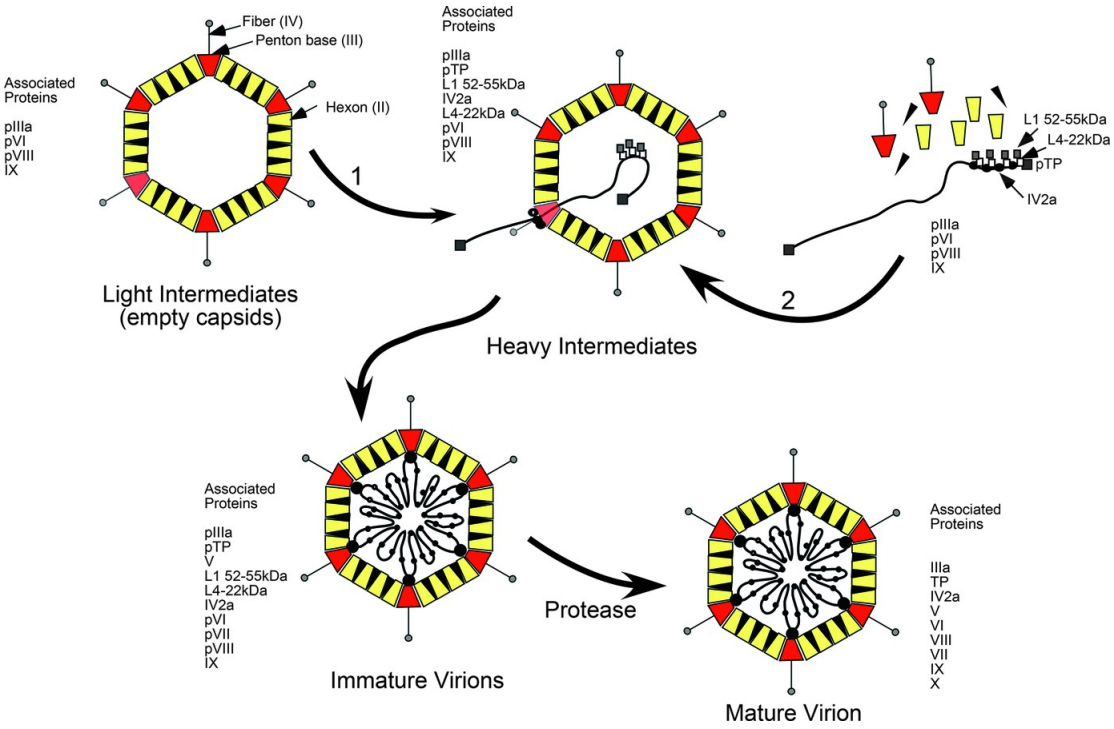

FIGURE 6.6 **Assembly pathway for adenoviruses**. Following transport into the nucleus, the hexons and pentons are proposed to assemble into empty capsids (previously known as light intermediates of assembly) around scaffolding proteins. These nonstructural scaffolding proteins are lost on packaging of viral DNA, which is inserted into this structure via a packaging sequence at the left end of the genome. The mechanism of insertion appears to be similar to DNA phages and involves a portal complex. The pIV2a protein may represent the ATP-hydrolyzing component required to drive DNA through the portal structure in the procapsid. Heavy intermediate forms of the capsid probably represent those in which DNA packaging is incomplete and the DNA is fragmented. Precursor core proteins would be packaged into the empty capsid along with the genome to form immature virions. Proteolytic cleavage of the precursor proteins by the viral proteinase yields the mature virion.

For a nonenveloped virus such as adenovirus, which replicates exclusively in the nucleus, there is a strong dependence on nuclear targeting/transport pathways to export newly synthesized mRNAs out of the nucleus and to import structural proteins back into the nucleus. Nuclear import of the major capsid protein, hexon or polypeptide II, depends upon the involvement of a second adenovirus protein, the pVI (precursor) polypeptide, which acts as a nucleocytoplasmic shuttling adapter and provides the necessary NLS for transporting the hexon into the

nucleus.[285] Trimer formation is in turn dependent upon yet another virus-encoded, chaperone–like protein, L4 100K, which transiently binds to the newly synthesized hexon monomer and mediates its association with two additional monomers.[33] Thus, the most abundant structural protein of the adenovirus capsid needs to interact with two additional virus-encoded factors in order to attain the correct tertiary structure and subcellular location for assembly.

The two other proteins that form the 12 vertices of the capsid, penton and fiber, appear to assemble independently in the cytoplasm. Mutations in the C-terminus of the penton that block assembly into pentamers do not prevent transport into the nucleus,[121] indicating that each monomer has an active NLS. In contrast, the fiber must form trimers to be efficiently transported to the nucleus,[187] even though this protein has an active NLS located at its N-terminus. It seems likely that the penton base and fibers are transported independently into the nucleus and assemble into the intact penton at the site of assembly.

Although two distinct pathways for adenovirus capsid assembly have been postulated from a large body of work in this area, it now appears that an empty procapsid is first formed around scaffolding proteins, in a manner similar to that observed with DNA phages.[3] Results from experiments that combined kinetic labeling with temperature-sensitive replication mutants and additional site–directed mutants have yielded the assembly scheme shown in Figure 6.6. A number of viral products appear to act as scaffolding proteins around which shells are assembled and which facilitate the encapsidation process. These proteins are present in the intermediate capsidlike particles but are absent once DNA has been encapsidated.[52] Disruption of adenovirus capsids with denaturants results in the release of groups of nine hexons that are associated with the faces of the icosahedron and which lack the peripentonal hexons. Under acidic conditions, these nanomers can reassemble to form icosahedral shells that lack the 12 vertices, which would normally be composed of the penton and the five peripentonal hexons, raising the possibility that hexon nanomers are intermediates of adenovirus capsid assembly. Following procapsid assembly, DNA and associated core proteins are then subsequently packaged into these empty shells to yield “immature virions” that then undergo proteolytic maturation through the action of the viral protease. Recent studies have demonstrated that the ~6-8 copies of the pIV2a protein, which binds to the packaging sequence, are present at a single apex of the mature virion.[40] This, and the fact that the pIV2a protein contains motifs (the Walker A and B boxes) associated with the binding and hydrolysis of ATP, suggests that it may represent the ATP-hydrolyzing component required to drive DNA through a portal structure in the procapsid. Consistent with this, mutations that either prevent synthesis of pIV2a or prevent binding of ATP to the protein block genomic packaging and result in the assembly of empty procapsids.[193]

Assembly of Enveloped Viruses in the Nucleus

Two enveloped animal virus families, the herpesviruses and the orthomyxoviruses, utilize cell components that are located within the nucleus in their replication and initiate their assembly within that compartment. In addition to importing into the nucleus the necessary components for assembly, these viruses must also export large nucleoprotein complexes back out into the cytoplasm.

In some respects, the herpesviruses represent a hybrid between a nonenveloped virus, such as adenovirus, and a more conventional enveloped virus such as a retrovirus, in that they utilize the compartments of the secretory pathway to transport and envelop large capsid structures from the nucleus to the outside of the cell. Members of this group of large viruses follow a complex assembly pathway in which genomic DNA is first replicated and encapsided in the nucleus. There they assemble an icosahedral capsid shell that is 160 Å thick and 1250 Å in diameter. The major component of this protein shell is pUL19, which forms both the pentameric and hexameric capsomeres necessary to assemble the icosahedral structure. Associated with the outer surface of the hexamers is the abundant small protein pUL35. Two additional proteins, pUL38 and pUL18, in a 1:2 ratio form heterotrimeric triplexes, which fit between and link together adjacent capsomeres.[179] Scaffolding proteins are essential for HSV-1 capsid assembly/maturation and in their absence incomplete and aberrantly shaped capsids are assembled. As with adenovirus, the major capsid protein lacks a nuclear targeting signal, and its transport into the nucleus requires an interaction with either the scaffolding protein (pUL26.5) or the triplex protein pUL38, which presumably provide the necessary NLS. pUL18 similarly requires pUL38 for nuclear localization, while pUL35 appears to be directed there via its interaction with pUL19.[217]

Studies of virus-infected cells together with *in vitro* assembly studies have provided valuable insights into the assembly process.[160,181] These studies point to a pathway in which pUL19, pUL38, and pUL18 assemble around a scaffold to form an icosahedral but predominantly spherical procapsid—the “B-capsids” identified by electron microscopy. Although not required for capsid formation, in its presence the portal complex apparently initiates capsid formation leading to its incorporation into the nascent capsid.[180] Cleavage of the scaffolding protein at a site near its C-terminus by the viral protease removes a 25-amino acid sequence that is necessary for binding to pUL19. The resulting disassociation and release of scaffold allows for packaging of the viral DNA genome and induction of maturation of the capsid into a more angular icosahedral structure, the previously identified “C-capsids.” DNA enters the procapsid through a unique vertex composed of the portal protein pUL6, which is assembled into rings composed of 12 subunits to form the portal complex.[182] The pUL6 portal resembles the connector or portal complexes employed for DNA encapsidation by double-stranded DNA bacteriophages such as φ29, T4, and P22. In the absence of an active proteinase, the scaffolding proteins remain associated with the protein shell preventing packaging of the viral DNA and the procapsid is unable to mature.[183]

Unlike adenoviruses, which accumulate in the nucleus and are released on lysis of the cell, herpesviruses exit the nucleus by a process termed nuclear egress, that first involves capsid budding into the lumen of the nuclear membrane. This process is dependent on the products of two highly conserved genes, UL31 and UL34, which encode a phosphoprotein and a type II membrane protein respectively. Nuclear localization of pUL31 is dependent upon its interaction with pUL34.[124] These two proteins form a heterodimer that represent the nuclear egress complex (NEC).[213] Although both proteins are present in the primary enveloped virions present in the lumen of the nuclear membrane, they are absent from mature virions, consistent with a model in which herpesvirus virions are first enveloped at the inner nuclear membrane, de-envelop by budding through the outer nuclear membrane, and are re-enveloped in the cytoplasm.[68] In the first step of nuclear egress, capsids bud at the inner nuclear membrane to form perinuclear enveloped virions (PEVs) in the perinuclear nuclear space. PEV formation involves localized disruption of the nuclear lamina, docking of capsids at and deformation of the inner nuclear membrane to form a nascent bud, and pinching off of the bud to release PEV. These are distinct from mature enveloped virions in having a smooth membrane and thin tegument layer.[92] PEV formation is subject to regulation since DNA-containing capsids are preferentially enveloped, likely due to specific interactions with UL31.[9] In order for capsids to access the cytoplasm, the PEV must undergo de-envelopment, losing their envelopes and NEC coats following fusion with the outer nuclear envelope. Several aspects of this process remain obscure, including how PEV target the outer rather than the inner nuclear membrane and which viral protein mediates membrane fusion.[68] Re-envelopment of cytoplasmic capsids occurs by budding into specialized vesicles that appear to be derived from multiple organelles, including the trans-Golgi network and early endosomes.[6,142] The complexities of

this latter interaction and subsequent steps in assembly will be discussed later in this chapter.

In order for influenza virus to take advantage of its unusual capacity to "steal" the capped 5′ ends of host cell mRNAs to initiate its own mRNA synthesis, transcription and viral RNA replication must occur in the nucleus (see Chapter 5). Genomic (minus sense) RNAs are replicated by a different mechanism to yield templates for mRNA synthesis as well as progeny viral genomes. The eight viral RNA segments of this virus are packaged into individual RNPs containing the four proteins of the transcriptase complex (PB1, PB2, PA, and NP, each transported into the nucleus by a functional NLS sequence) and the nuclear export protein (NS2/NEP) but are not exported into the cytosol until late in infection when the viral matrix protein (M1) begins to be synthesized.[146] It has been shown that the vRNPs remain in the nucleus of cells if either M1 or NS2/NEP is not synthesized. NS2/NEP does not interact directly with vRNPs, rather it mediates (RanGTP-dependent) formation of a bridge between the cellular export receptor Crm1 and the N-terminal domain of M1, which in turn binds to the vRNP via its C-terminal domain. This "daisy-chain" complex of (Crm1-RanGTP)-NEP-M1-vRNP is likely what mediates the export of vRNP across the nuclear envelope.[4,177] M1 association with the vRNPs also appears to be important for preventing their re-entry into the nucleus, likely by masking the NLS on the component proteins.[67] Thus, the matrix protein of influenza virus is a key modulator of vRNP transport into and out of the nucleus.

Assembly of Viruses in the Cytoplasm

While targeting and import of proteins into the nucleus or secretory pathway involves well-characterized motifs on the proteins involved, the processes by which proteins are targeted to destinations within the cytoplasm remain less well defined. Most viruses, even those that are nonenveloped, initiate or complete their assembly in association with membranes of the secretory or endocytic pathways. Association of viral proteins with membranes acts to enrich viral proteins or genomes in specific cellular domains to increase the efficiency of assembly steps and organizing the different viral process.

Intracytoplasmic Transport and Assembly of Retroviral Capsids

Retroviruses are enveloped viruses that, for the most part, complete their assembly by budding through the plasma membrane of the infected cell. For these viruses, the immature capsid of the virus is assembled from polyprotein precursors that must be transported through the cytoplasm to the inner leaflet of the membrane. The viral glycoproteins, on the other hand, must be transported through the secretory pathway of the cell to the cell surface where they colocalize with the nascent, membrane-extruding capsid (Fig. 6.7). All replication competent retroviruses contain four genes that encode the structural and enzymatic components of the virion. These are *gag* (capsid protein), *pro* (aspartyl proteinase), *pol* (reverse transcriptase and integrase enzymes), and *env* (envelope glycoprotein). However, the product of the *gag* gene has been shown to possess the necessary structural information to mediate intracellular transport, to direct self-assembly into the capsid shell, and to catalyze the process of membrane extrusion known as budding.[230] For most retroviruses, the nascent Gag polyproteins are transported to the plasma membrane where assembly of the capsid shell and envelopment occur simultaneously (Fig. 6.7, pathway 1). Viruses that undergo this "type-C" form of morphogenesis include members of the alpha- and gammaretroviruses. Lentiviruses and deltaretroviruses assemble their capsids in a similar fashion in most cell types. In the second morphogenic class of retroviruses, the "type B/D" class, the Gag precursors are targeted first to an intracytoplasmic site where capsid assembly occurs. These assembled immature capsids are then transported to the plasma membrane where they undergo budding and envelopment (Fig. 6.7, pathway 2). Viruses that undergo this process of assembly and release include members of the betaretroviruses. Members of the spumavirus family also assemble immature capsids in the cytoplasm, but they are targeted to the ER or ERGIC for envelopment (Fig. 6.7, pathway 3).

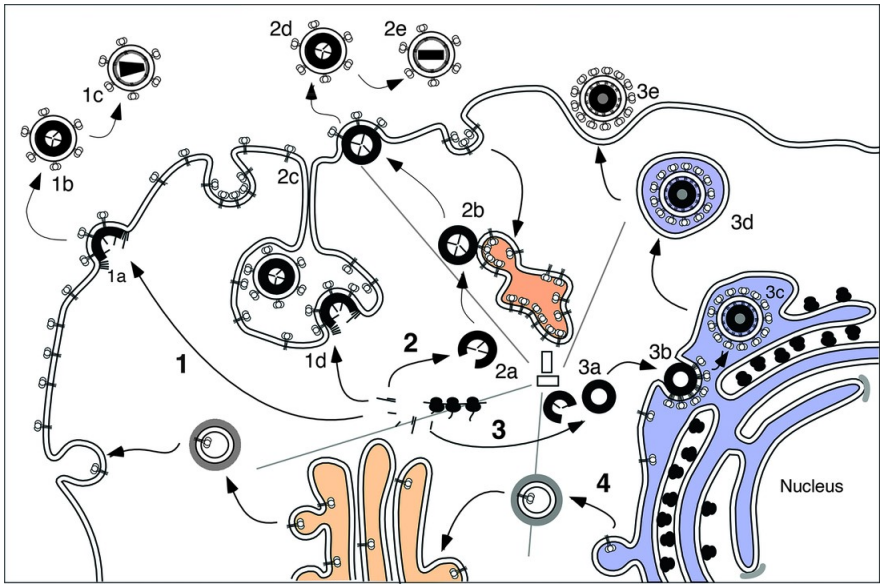

FIGURE 6.7 **Assembly of retroviruses**. The assembly pathways of retroviruses that exhibit C-type morphogenesis (*pathway 1*), B-/D-type morphogenesis (*pathway 2*), and that of the foamy viruses (*pathway 3*) are shown. The envelope glycoproteins are translated on membrane-bound polysomes and for most retroviruses are transported to the cell surface through the cell's secretory pathway (*pathway 4*), although glycoproteins destined for incorporation into virions are likely endocytosed and interact with Gag intracellularly (*pathway 2b*). For all morphogenic classes, the Gag proteins are synthesized on free polysomes. In the case of the C-type morphogenic viruses (ie, RSV and HIV), the Gag and Gag-Pol proteins migrate either individually or in small multimers to the plasma membrane where immature capsid assembly and envelopment occurs concurrently (*1a*). At some point in this pathway, the viral genomic RNAs associate with the Gag and Gag-Pol precursors and are incorporated into the developing capsid. For HIV in macrophages assembly can occur on deep invaginations of the plasma membrane to which Env has been targeted (*1d*). In the case of the B-/D-type viruses, polysomes translating Gag and Gag-Pol precursors are first transported via microtubules to an intracytoplasmic, pericentriolar assembly site (*2a*) where they

assemble into immature capsids. The immature structures are then transported, most likely in association with endosomal vesicles (*2b*), to the plasma membrane (*2c*) where they associate with the envelope glycoproteins and induce viral budding. For both classes of retroviruses, the capsids of the nascent immature particles appear as doughnut-shaped structures and contain unprocessed Gag and Gag-Pol precursors (*1b and 2d*). The mature virus particles contain electron-dense cores with morphologies characteristic of the virus (*1c and 2d*). The maturation step is required for infectivity and is the result of the activation of the viral protease, which cleaves the Gag and Gag-Pol precursors into the internal structural and enzymatic proteins of the virus. For the foamy viruses, Gag and Pro-Pol precursors also assemble into immature capsids in a pericentriolar site (*3a*), but budding primarily occurs at the ERGIC compartment where the viral glycoproteins are retained (*3b*). The enveloped virion is presumably transported to the plasma membrane by transport vesicles (*3d*). Maturational cleavage of the immature core is limited to removal of 4 kd from the C-terminus of the precursor; the mature infectious virion maintains an immature morphology (*3e*).

While the size and protein content of the precursor varies between different retroviral families, at least three *gag*-encoded proteins are found in all retroviruses; these are the matrix protein (MA), the capsid protein (CA), and the nucleocapsid protein (NC). In addition to these functionally conserved domains, the Gag precursor can, depending on the virus encoding it, contain additional peptide sequences (Fig. 6.8) whose functions in virus assembly and future cycles of infection are only now being resolved.

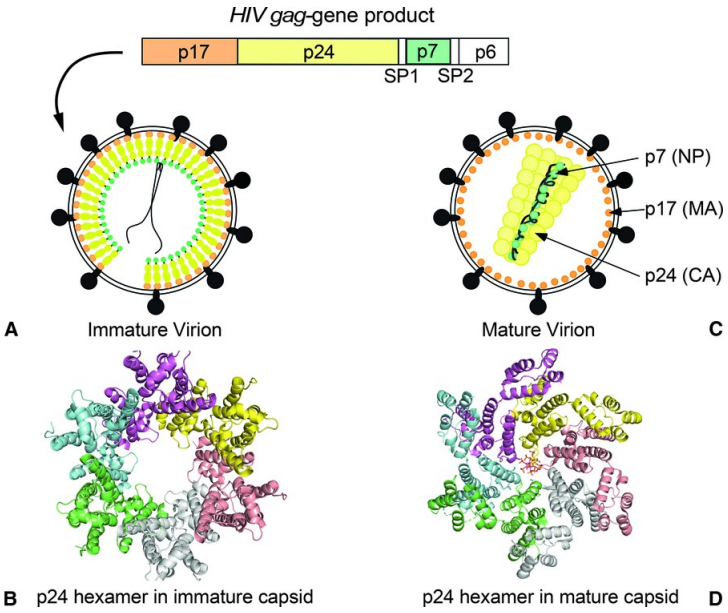

FIGURE 6.8 **Immature and mature HIV virions with p24 hexameric rearrangement 2022**. The Gag precursor polyproteins of all retroviruses contain beginning at the N-terminus, matrix (MA), capsid (CA), and nucleocapsid (NC) domains linked in this order. The *gag* gene of the lentivirus human immunodeficiency virus (HIV) is shown. The specific name associated with the Gag cleavage products is derived from their respective apparent molecular weights ($\times 10^{-3}$). Immature virions **(A)** are assembled from Gag and Gag-Pro-Pol precursor proteins arranged in hexamers **(B)** primarily through molecular interactions of the p24 domain that stabilized by the SP1 domain, which forms a six-helix coiled-coil structure. Following proteolytic cleavage of the precursor to its component proteins, a major structural rearrangement occurs to form the conical core **(C)** comprised of a more tightly spaced p24 hexamer that is now stabilized by interactions between the NTD and the small molecule, inositol 6-phosphate **(D)**.

The molecular details by which the capsid precursor proteins are directed to the site of assembly are incomplete, but the process appears to be mediated primarily by the MA domain of the Gag precursor. In most retroviruses, the matrix protein contains two elements involved in plasma membrane targeting. The first of these is an N-terminal myristic acid, which is normally sequestered but can be induced to insert into the hydrophobic lipid bilayer via a myristyl switch.[228] The second is a surface patch of basic amino acids that mediate the initial interaction of Gag with the negatively charged, phospholipid head groups of the membrane. Mutations that interfere with either myristylation or the charged residues can abrogate plasma membrane targeting, and in some instances the mutated Gag precursors are targeted to internal membranes.[191]

In the betaretroviruses, where capsid assembly and virus budding are discrete events, a genetic dissection of this process has shown that Gag-containing precursors express a dominant sorting signal (the cytoplasmic targeting/retention signal or CTRS) that targets the proteins to the initial assembly site.[39] Studies suggest that the CTRS interacts with Tctex-1, a light chain of the microtubule-associated dynein motor, and directs nascent Gag proteins and translating polysomes to the centriolar region of the cell where immature capsid assembly occurs.[243,275] Efficient transport of these capsids out of the assembly site is dependent on both the presence of the M-PMV Env protein and endosomal trafficking and appears to reflect a requirement for Gag-Env interactions at the pericentriolar recycling endosome.[203] It is likely that similar preassembly complexes also participate in the intracellular transport of human immunodeficiency virus-1 (HIV-1) Gag proteins since their transport is modulated by the cellular adaptins AP-1, AP-2, and AP-3, and intracellular interactions with Env direct Gag assembly to specific plasma membrane regions of the cell.[10,62] Although in macrophages HIV-1 Gag was initially thought to be targeted to a late endosomal compartment for intracellular assembly and budding, more recent studies suggest that these compartments are derived from deep invaginations of the plasma membrane (Fig. 6.7, pathway 1d).[10] Targeting by HIV-1 Gag for assembly in part reflects recognition of enriched regions of phosphotidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) in the plasma membrane by the highly basic MA domain. Depletion of this lipid component by overexpression of the cognate phosphatase (5-phosphatase IV) redirects HIV-1 assembly away from the plasma membrane to internal membranes.[190]

Irrespective of the assembly site, Gag precursor proteins must associate in a reproducible fashion to assemble into a nascent immature capsid. Mutational analyses of *gag* genes, as well as *in vitro* assembly studies, have shown that the CA and NC domains of Gag play a critical roles in

assembly; MA is dispensable for this process.[49,77] NC binding to RNA may act to nucleate the capsid assembly process, while CA, which is the key structural component of the immature capsid and of the conical mature viral capsid, forms a symmetrical hexameric network of proteins. CA is comprised of two major domains, an N-terminal domain (CANTD) connected through a short flexible linker to the C-terminal domain (CACTD), with the latter required for capsid dimerization and Gag oligomerization. Cryoelectron tomography studies of immature HIV-1 capsids have revealed a curved hexameric lattice with a radial distribution of Gag from the membrane-associated N-terminal MA region to the internal C-terminus (Fig. 6.8). Gag hexamers are stabilized by SP1, a region C-terminal to CA which forms a six-helix bundle, which in turn is stabilized by a small molecule, inositol hexakisphosphate (IP6).[65,130] A striking observation in the context of HIV-1 is that released immature virus particles have an incomplete protein shell, with the ordered Gag lattice covering, on average, only two-thirds of the membrane surface.[28,287]

For most retroviruses, capsid assembly drives the process of membrane-extrusion known as budding. As we will discuss below, in order for an infectious virus to be formed, the Gag precursors (or for other viruses, NPs) and surface glycoproteins of the virus must be targeted to the same region of the same membrane. In this way, during virus budding, a proper complement of glycoproteins can be incorporated into the nascent virion.

Assembly of Enveloped Viruses at Cellular Membranes

For most enveloped viruses, the location within the cell at which envelopment takes place is determined by the targeting to or retention of the viral glycoproteins at that site. Indeed, with the exception of the retroviruses, the efficiency of virus budding and particle release is highly dependent on the presence of the envelope glycoprotein(s), and in its absence few particles are produced. Since the glycoproteins define the site of virus budding, specific interactions between the viral NP and the glycoproteins, sometimes mediated by a matrix protein, must take place to drive bud formation and ensure that the genome of the virus is incorporated. For each of the viruses that assemble at intermediate points within the secretory pathway, fully assembled viruses must traverse the remainder of the pathway in order to be released from the cell (Fig. 6.9). Glycoproteins on these released viruses have complex oligosaccharides and are most likely modified by the Golgi-localized enzymes on the way to the cell surface.

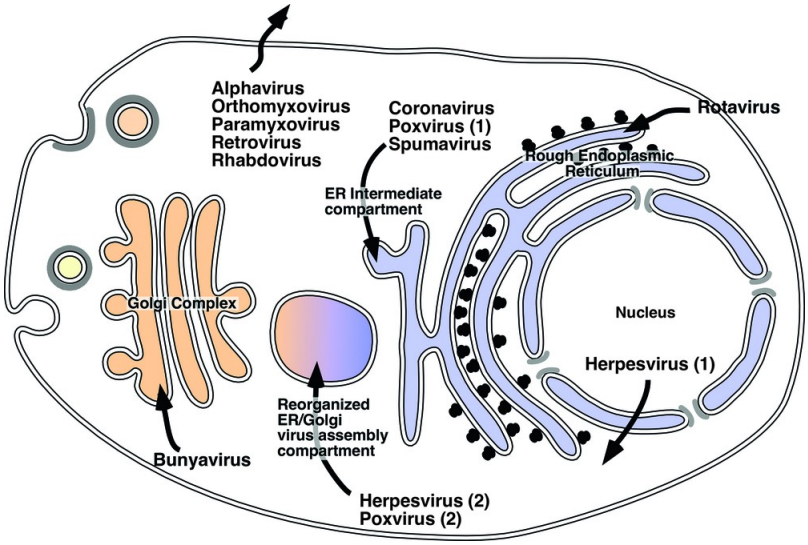

FIGURE 6.9 **Viral assembly at cellular membranes**. Schematic representation showing the intracellular locations at which enveloped virus assembly takes place. For each virus that is enveloped at organelles within the secretory pathway, the virions must traverse the remainder of the pathway to be released from the cell. Rotaviruses appear to utilize the ER membrane as a scaffold for assembly of virion proteins—the capsids that form during the assembly process are only transiently enveloped and nonenveloped particles accumulate in the lumen of the ER. Coronaviruses localize the three membrane proteins (E, M, and S) in the ER-Golgi intermediate compartment and virus budding occurs into the lumen of this compartment of the secretory pathway. In contrast, the vaccinia virus nucleocapsid appears to be wrapped by a double membrane derived from this compartment (poxvirus (1)) but remains free in the cytoplasm so that it can then be further enveloped by a reorganized membrane compartment derived from ER and Golgi-derived membranes (poxvirus (2)). Similarly, herpesviruses initially bud into the lumen of the nuclear membrane (herpesvirus (1)), but after fusion and release of the capsid into the cytoplasm, it is re-enveloped by membranes from a reorganized ER-Golgi–derived assembly compartment (herpesvirus (2)). The G1 and G2 glycoproteins of bunyaviruses colocalize in the trans-Golgi compartment to direct budding of nucleocapsids at this location. For the retroviruses, which can assemble and release virus particles in the absence of envelope glycoproteins, the envelope glycoproteins appear to direct virus budding to the basolateral plasma membrane of polarized epithelial cells.

Assembly at the Endoplasmic Reticulum

For positive-stranded RNA viruses from the *Flaviviridae* family, genome replication and assembly of virus particles occurs on specialized ER membranes (Fig. 6.10). Within this group, infection by members of the *flavivirus* genus, including dengue virus and Zika virus, leads to a reorganization of cellular ER-derived membranes to form vesiclelike compartments within the ER leaflet that facilitate virus replication and act to initiate assembly events on adjacent membranes.[48,86,284] Viral genomic RNA is transported out of the replication vesicles and directed either toward translation or assembly, likely dependent on the abundance or availability of viral structural proteins. Initiation of the assembly process is orchestrated by the viral nonstructural protein NS2A, which is involved in the proteolytic processing of the structural proteins and chaperoning of viral RNA.[288] To initiate assembly, the polyprotein precursors composed of capsid (C) and the premembrane protein (prM) are proteolytically cleaved both on the cytosolic and luminal sides of the membrane to release capsid from the membrane and allow the glycoproteins to form trimeric spike structures.[14] This process is coordinated by NS2A and is facilitated by the viral protease NS2B/3 protease

in the cytosol and host peptidases in the ER. Once released from the membrane, capsid protein can form dimers that associate both with the viral RNA genome and glycoprotein cytosolic domains. Delivery of the viral genome to the site of assembly appears to also be coordinated by NS2A through interactions with the 3'UTR region of the genome. The viral glycoproteins are maintained at the site of assembly in the ER through a cytosolic ER retention sequence in the viral envelope (E) protein.[104] Virion assembly is initiated by the viral C protein, which forms bridging interactions between the viral genomic RNA and the membrane glycoproteins, prM and E. These interactions are thought to stabilize the capsid oligomers to drive budding into the ER membrane. The lack of visible preassembled C proteins suggests that encapsulation and budding occur simultaneously.

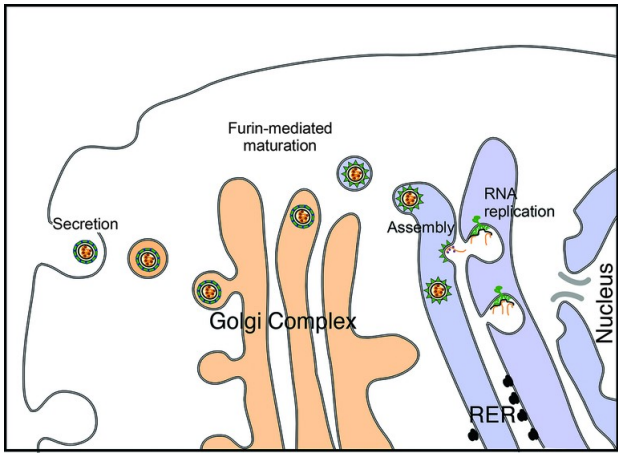

FIGURE 6.10 **Flavivirus assembly**. Flavivirus genome replication occurs in specialized vesicles that form within ER membranes. Virus assembly sites form on adjacent ER membranes through interactions between virus structural proteins and newly synthesized genomic RNA. Virions bud into ER membranes in a process simultaneously with nucleocapsid formation. Once in ER lumen, immature virus particles traffic to the Golgi complex where the prM glycoprotein is cleaved by the host furin protease to form mature virus particles. Mature virions travel to the plasma membranes in secretory vesicles where they are released into the extracellular space.

Hepatitis C virus, another member of the Flaviviridae family, also buds into ER membranes, but *de novo* viral particles are secreted through lipid export pathways rather than the normal secretory system.[244] At later stages in the HCV infection cycle, viral components required for assembly, including the HCV capsid protein, core, the viral envelope glycoproteins, nonstructural NS5A, and genomic RNA, are recruited to virus assembly sites surrounding lipid droplets (LDs).[164] NS5A interacts with viral RNA and is thought to be involved in recruitment of the viral genomes from the site of replication to assembly complexes through interactions with the LD-associated proteins. Accumulation of viral factors on the surface of LDs is accompanied by rearrangements in ER membranes to surround LDs, forming membrane-wrapped assembly sites.[129] This creates an environment that is highly enriched for both the viral and host lipid factors required for assembly. HCV core proteins associate with both viral RNA and envelope proteins, which are thought to facilitate budding into the ER. Although the precise location of budding within this assembly site and kinetics of egress are still unclear, it has been shown that the VLDL export pathway is required to produce highly infectious virus particles and that extracellular particles exist as lipid hybrid structures associated with apolipoproteins.

Assembly at the Endoplasmic Reticulum-Golgi Intermediate Compartment

The coronaviruses are positive-stranded RNA viruses with large (30 kb) genomes packaged in a helical nucleocapsid. The nucleocapsid acquires its envelope by budding into intracellular organelles including the ERGIC, a pre-Golgi compartment of the secretory pathway, or lysosomes. Coronaviruses invariably encode three envelope proteins, each of which contain membrane targeting sequences that drive the localization of assembly to specific cellular membranes. The spike protein (S), which determines the host-range of the virus, is a type I glycoprotein that forms the distinct bulbous peplomers of the virus. S proteins from several coronaviruses are transported to the plasma membrane, although they contain both ER retention and endocytosis signals that can redirect it to the ERGIC.[63,112,138,229] In the case of SARS coronaviruses, protein localization to the cell surface can cause cell to cell fusion events leading to syncytia formation. The most abundant virion protein is the membrane (M) glycoprotein. M spans the lipid bilayer three times exposing a short N-terminal domain outside the virus and a long C-terminus inside the virion. The small envelope protein (E), which is only a minor component of virions, is the key to defining the site and nature of coronavirus envelopment.[101] E is a hydrophobic type 1 membrane protein that localizes to the ERGIC and induces the formation of tubular, convoluted membrane structures characteristic of virus infection.[47,210] The E protein of the avian coronavirus infectious bronchitis virus (IBV) appears to be retained in the ERGIC by a novel ER retrieval signal (RDKLYS-COOH) and localizes M to this site through intermolecular interactions.[133] Combined expression of the three membrane structural proteins E, M, and S increases the amount of S and M proteins retained in the ERGIC, suggesting that a combination of cellular targeting signals and intermolecular interactions between viral proteins are required to determine the assembly site.[270]

For most coronaviruses, coexpression of E, M, and S results in the assembly and release from cells of viruslike particles (VLPs) containing these three viral membrane proteins.[270] In the case of SARS coronaviruses, this process also requires the nucleocapsid (N) protein for efficient VLP formation.[25,247] The enveloped VLP form a homogeneous population of spherical particles indistinguishable from authentic virions in size and shape.[274] Only M and E are required for efficient particle formation and expression of E alone can mediate particle release.[143] The S glycoprotein is thus dispensable for virus particle assembly but is retained in the Golgi by the M protein, which appears to direct its assembly into virions. In infected cells, S protein trimers are observed to be enriched on the luminal side of budding membranes at the ERGIC assembly sites.[123,157] On the cytosolic side of membrane, M protein directs the incorporation of nucleocapsids containing the genome length RNA into virions at the budding site in ERGIC membranes.[175]

Spumaviruses are another class of viruses that assemble in ERGIC. Although a majority of the retroviruses are enveloped at the plasma membrane of the cell, for most members of the spumavirus genus, this occurs on internal membranes. In human fibrosarcoma cells, primate

foamy virus (FV) Gag and Env appear to colocalize predominantly in the trans-Golgi region of the cell,[293] but in other cells, immature capsids appear to bud into the ERGIC region of the secretory pathway. This is consistent with the observation that expression of the primate FV Env complex, gp80/gp48, in the absence of other structural proteins, results in its localization to the ER. A dilysine ER retrieval motif at the C-terminus of gp48 is responsible for this localization, and mutation of either lysine results in efficient transport of the protein to the PM. While a greater fraction of capsids bud from the PM in mutant virus–infected cells, envelopment of capsids at the ER is still observed.[87] A second distinguishing feature of foamy viruses is the dependence of capsid envelopment on Env expression.[11] As with the betaretroviruses, assembly of immature FV capsids is targeted to the pericentriolar region of the cell by a CTRS located around an arginine residue at position 50 in Gag.[293] They are then transported to the ER (or PM) for envelopment. In the absence of FV Env, these preassembled capsids do not associate with membranes or initiate budding. Recent experiments have shown that it is the posttranslationally cleaved (148 amino acids long), membrane-spanning, signal peptide domain of the foamy virus Env that mediates capsid membrane association/envelopment and that this protein is incorporated into the virus in the process.[135]

Assembly in the Golgi Complex

Bunyaviruses are negative-stranded, enveloped viruses with a segmented genome that assemble in tubelike virus factories that are built around the Golgi complex and are connected to mitochondria and RER Figure 6.9, reviewed in Ref.[279] By physically juxtaposing viral RNA replication and assembly, these factories appear to allow accumulation of RNPs that can associate with viral glycoproteins and bud into the lumen of swollen Golgi stacks. In general, bunyaviruses encode two type I glycoproteins Gn (previously G1) and Gc (G2) that are translated as a polyprotein precursor together with the nonstructural protein NSm.[105] Gn and Gc are cotranslationally cleaved from the precursor protein by signal peptidase, which cleaves after the internal signal sequence that mediates translocation of Gc. For bunyaviruses from the *Nairoviridae* family, additional cleavage events by furinlike or SKI-1/S1P-like proteases are required for Gn and Gc maturation. The two glycoproteins have been shown to fold with distinctly different kinetics, but once properly folded, form a Gn-Gc heterodimer that is transported to the Golgi complex where virus budding occurs. Gc expressed in the absence of Gn is retained in the ER, while Gn expressed alone is targeted to the Golgi reviewed in Ref.[93] The Uukuniemi virus Golgi localization signal of Gn has been mapped, through analysis of mutations and glycoprotein chimeras, to the membrane proximal half of the 98 amino acid long cytoplasmic tail of the protein. Glycoprotein retention in this case appears, therefore, to depend on interactions between the cytoplasmic tail of Gn in the Gn-Gc heterodimer with components residing on the cytoplasmic side of the Golgi membrane. However, while all bunyavirus Gn-Gc complexes accumulate in the Golgi, the exact location and nature of the signal(s) that ensure this do appear to differ among the genera.[279]

During assembly of bunyaviruses, the helical ribonucleoproteins, consisting of the three single-stranded genomic RNA segments and the associated nucleocapsid (N) protein and the viral RdRp, accumulate in the Golgi component of the virus factories and through interactions with the Gn and Gc cytoplasmic domains initiate the budding of virus particles into the Golgi lumen. The region of the secretory pathway at which Gn-Gc heterodimers accumulate clearly defines the budding site since in the presence of brefeldin, a drug that redistributes Golgi components to the ER, virus budding occurs into the ER.[93]

Assembly at the Plasma Membrane

Members of several virus families undergo their envelopment at the plasma membrane. These families include the togaviruses, the rhabdoviruses, the para- and orthomyxoviruses, and the retroviruses. In each of these cases, the viral glycoproteins, either as hetero-oligomeric complexes or homo-oligomers, have traversed the entire secretory pathway to be delivered to the plasma membrane of the cell. Assembly at the plasma membrane obviates the need for the assembled virus to navigate additional compartments of the secretory pathway, since virions are released directly into the external milieu of the cell. Alphaviruses are among the best characterized of these different viral systems since a combination of biochemical, genetic, and structural information has been amassed to shed light on the complexity of this assembly process. The major glycoproteins E1 and E2 of the alphaviruses are translated from a subgenomic 26S RNA as a p62, 6K, E1 precursor complex. The 6K and E1 proteins are released from the precursor by signal peptidase but remain in a complex with p62. This complex protects E1 from low pH during trafficking until, following transport to the Golgi, p62 is processed to E2 and E3 by the host furin protease.[234] Stable trimers of E1-E2 heterodimers are then transported to the PM, where they form a lattice that associates with nucleocapsids formed from the capsid (C) protein and genome length RNA. Although not strictly required for budding, the 6K protein as well as the viral TF protein, produced through a ribosomal frameshift in the 6K coding region, greatly enhance the process of alphavirus assembly.[234] The 6K and TF proteins travel to the PM with the E1-E2 complex, but only the palmitylated TF protein is incorporated into virions.[114,211] At the plasma membrane, budding occurs at specialized membrane domains that are characterized by accumulation of E2 and the absence of host proteins.[82]

Cryo-EM analyses of mature alphavirus particles revealed a detailed structure of this enveloped virion.[144,170] These studies have shown that both the envelope as well as the core display icosahedral symmetry. Surprisingly, however, the trimers of E1 and E2 are located at the threefold and quasi-threefold symmetry axes of the pentameric and hexameric order of the nucleocapsid. Moreover, the heterodimers of each spike splay out above the membrane in a skirtlike fashion, traverse the lipid bilayer individually, and interact with three underlying capsid (C) proteins that belong to three separate capsomeres. This creates a complex network of molecular interactions where the glycoprotein-capsid protein interactions mediate not only the binding of the nucleocapsid to the spikes but also stabilize the connections between the capsomeres.[114] Above the membrane, the skirts formed by the E1-E2 heterodimers form lateral connections that mimic the pentameric and hexameric arrangement of the capsid. These interactions may facilitate the process of budding by providing a multivalent binding site for the capsid, as well as by providing a force for membrane bending. Budding, however, requires a cooperative interaction between the glycoproteins and the capsid protein, since in the absence of either budding does not occur. Moreover, mutations in the cytoplasmic tail of the E2 protein alone can abrogate budding. Two models have been proposed for assembly.[82] In the first, glycoprotein trimers assemble around a preformed nucleocapsid that is associated with the inner leaflet of the PM. This model is supported by observations of abundant preformed nucleocapsids in the cytosol of infected cells and through microinjection of *in vitro* assembled nucleocapsids that can induce budding.[36,251] The second hypothesizes that it is through interactions between E1 and E2 heterotrimers that the icosahedral structure of the virus is established and that interactions between the E1-E2 heterodimers and C proteins organize the nucleocapsid similarly. Evidence for the latter model came from deletion mutants in C that are defective in the formation of intracellular capsids but which nevertheless can associate with E1-E2 to facilitate the release of T = 4 icosahedral capsids.[76]

For the negative-strand RNA viruses, orthomyxoviruses, paramyxoviruses, and rhabdoviruses, an additional protein, the matrix protein, mediates the interactions between the viral glycoproteins and the RNP and appears to play a key role in envelopment. These proteins are able to

bind to membranes through hydrophobic domains or, as with the retroviruses, through a cluster of positively charged residues that initiates electrostatic interactions with the PM. Cross-linking studies have demonstrated that these M proteins form homo-oligomers in the virus and can self-associate *in vitro* or when expressed at high levels in cells. As discussed earlier, the M1 protein of influenza virus acts to mediate the transport of RNPs from the nucleus to the site of virus assembly on the plasma membrane. After nuclear export, M1-vRNP complexes associate with Rab11a-containing vesicles in the perinuclear region, which appear to move along cytoskeletal components of the cell.[8] The importance of the M protein in the paramyxovirus budding process was inferred from the defective measles viruses found in subacute sclerosing panencephalitis that are unable to assemble virus particles and that have mutations in the M protein coding region.[19] The development of reverse genetics systems for the negative-stranded viruses, in which mutations can be reintroduced into the viral genome, has allowed a more rational approach to examining these questions. Construction of rhabdovirus genomes that lack the M coding region resulted in a dramatic ($>10^5$-fold) decrease in the release of virus particles and those that were released lacked the characteristic bullet-shape morphology. The defect could be complemented by expression of M in trans.[154] Thus, in the rhabdoviruses, the M protein condenses the helical RNP into its characteristic shape and mediates the envelopment process. Similarly, for the orthomyxoviruses, M proteins can define the shape of the virus particles,[26] and while in the absence of M1 particles can be released, this is a much less efficient process and the resulting virus particles are less infectious.[225]

Reverse genetics approaches have also shed light on the role of the G glycoprotein in rhabdovirus assembly and of the hemagglutinin (HA) and neuraminidase (NA) in orthomyxovirus assembly. Rhabdoviruses that lack the G-protein coding domain do assemble and release bullet-shaped particles, but at only 3%-10% the efficiency of wild type.[153,220] Initial studies with G proteins lacking a cytoplasmic tail suggested that this domain might be important in the budding process; however, more recent studies have shown that while there is a general requirement for a short cytoplasmic tail, it is amino acid sequence independent and that G proteins with large C-terminal extensions can be efficiently incorporated into virions, thereby leaving the basis for M protein interactions with G unresolved (reviewed in Ref.[111]). While foreign glycoproteins can be efficiently incorporated into rhabdovirus particles (creating pseudotype particles), they do not stimulate budding.[218] This is consistent with experiments that have identified a domain within the extracellular membrane–proximal stem (GS) of vesicular stomatitis virus (VSV) G that is required for efficient VSV budding. Recombinant viruses encoding glycoprotein chimeras with 12 or more membrane-proximal residues of the GS, as well as the G protein transmembrane and cytoplasmic tail domains, produced near wild-type levels of particles. In contrast, those with shorter regions produced 10- to 20-fold fewer particles. It is possible, therefore, that this region of the G protein membrane proximal domain modifies the membrane to facilitate the budding process.[220] VSV assembly begins with the formation of an RNA-N protein nucleocapsid ribbon, which is then bound by M, P, and L proteins in an uncondensed state.[214] The RNP is then recruited to membranes enriched for M and G proteins, where it condenses, forming a tight ring that represents the tip of the bullet-shaped particle, which are then forced to curl into larger rings that eventually tile the helical trunk. The assembling structure is stabilized by the binding of M protein to the outside of the nucleocapsid, which in turn forms a triangular platform for binding G-trimers and the lipid membrane.[84]

In influenza virus, the fact that the cytoplasmic domains of all three membrane proteins (HA, NA, and M2) are highly conserved in all isolates of the virus, pointed to a role for these domains in assembly of enveloped virus. Early studies indicated that HA proteins with foreign cytoplasmic tails are not incorporated into virions, although HAs lacking this domain can be incorporated at levels 50% that of wild type.[173] Studies using reverse genetics have confirmed these findings and have shown that the short (6 amino acid) cytoplasmic domain of NA also plays an important role in morphogenesis. Viruses encoding the truncated NA protein were released less efficiently and were larger and more filamentous. However, in viruses encoding tail-less versions of both HA and NA a 10-fold reduction in virus release was observed and morphogenesis was drastically altered. Virus particles released from these cells were greatly elongated with an extended irregular shape and a reduced level of viral RNA. Similarly, truncation of the cytoplasmic tail of M2, which binds to M1, results in much reduced virus infectivity, coupled with reduction in the amount of packaged RNA and budding efficiency.[152] More recent studies have found a nonuniform distribution of HA and NA within virus particles, and this distribution, seen in budding membranes, is important for the formation of infectious virions, with proper glycoprotein distribution requiring M protein.[37] Thus, it appears that for influenza virus, interactions between M1, M2, and the viral glycoproteins proteins at the plasma membrane are all important for envelopment and morphogenesis.[176,225]

With the exception of the spumaviruses, glycoprotein-capsid interactions are not absolutely required for the assembly and release of enveloped retrovirus particles; the Gag precursor alone contains the information necessary to be specifically targeted to the plasma membrane and to drive budding. Nevertheless, for members of the lentivirus family, such as HIV and SIV, that encode envelope glycoproteins with a long (150+ amino acid) cytoplasmic domain, a specific interaction between this region of the transmembrane (TM) component of Env and the MA domain of the Gag precursor appears to be necessary for Env incorporation. For these viruses, mutations in MA or in the cytoplasmic tail can abrogate Env incorporation.[130] Further evidence for an Env-Gag interaction is derived from polarized epithelial cells, where retroviral glycoproteins define the PM domain at which virus budding and release occur (see below).

Several viruses appear to make use of cholesterol- and sphingolipid-rich domains on the plasma membrane known as lipid rafts to assist in the concentration and organization of viral components for assembly.[34] These rafts are characterized by their insolubility in detergent at low temperatures (4 °C), and proteins associated with them remain in the insoluble fraction following extraction from cells. Both HA and NA of influenza virus cluster in lipid rafts via targeting signals that have been mapped to both their transmembrane and cytoplasmic domains, and it is hypothesized that, through interactions with the clustered glycoproteins, M1 and the vRNPs are organized for budding.[177] Consistent with these observations, the lipid bilayer of influenza virions is enriched in cholesterol and sphingolipids and appears also to be in ordered domains.[237] Similarly, the glycoproteins and capsid precursors of HIV-1 have been shown to associate with lipid rafts, and depletion of cholesterol from the plasma membrane impairs HIV-1 particle production.[278] Gag-Env interactions appear to be required for Env association with lipid rafts, since mutations in either MA or the cytoplasmic domain of the gp41TM protein that interfere with Gag-gp41 interactions prevent Env association with rafts.[16] Although it was initially assumed that VSV assembled independently of rafts, it has been shown that G clusters in microdomains and can colocalize on the PM with proteins known to be located in rafts.[206] Thus, it is possible that VSV also utilizes rafts to organize its components for assembly.

Targeting of Viral Glycoproteins in Polarized Epithelial Cells

Many viruses initiate their infection of a host by interacting with cells at an epithelial surface. Individual cells within an epithelial layer are tightly connected by junction complexes that form a barrier to diffusion of molecules throughout the cell membrane and divide the cell surface into two distinct plasma membrane domains: the apical domain, which faces the exterior, and the basolateral domain, which faces the interior.

As a result of differential targeting of lipids and protein components to apical and basolateral membranes, epithelial cells in tight monolayers are highly polarized with each plasma membrane domain having a distinct lipid and protein composition.[222] The assembly and release of many viruses from epithelial cells is also highly polarized, occurring selectively at either the apical or basolateral surface. Influenza virus releases newly assembled virions from the apical surface of polarized epithelial cells, while VSV and many retroviruses are released from the basolateral membrane. In the absence of other viral proteins, VSV G protein and several retroviral Env proteins are transported to the basolateral surface, while influenza virus HA, NA, and M2 are targeted to the apical membrane.[45,176] Sorting of proteins to one or the other domain occurs in the trans-Golgi network, and recent evidence suggests that proteins destined for the basolateral surface contain within their cytoplasmic domain motifs that direct the protein to that membrane. In the VSV G protein, a tyrosine-based motif within the cytoplasmic tail appears to be critical for basolateral targeting.[266] Similarly, tyrosine-based endocytosis motifs in the cytoplasmic domain of both the MuLV and HIV Env proteins are important for their polarized expression and for basolateral budding of their cognate viruses.

Apical transmembrane proteins appear to contain two signals that probably act cooperatively in targeting to the apical surface. Glycosylation in the ectodomain and a signal in the membrane-spanning domain function together to ensure association with sphingolipid-cholesterol–enriched membrane domains or rafts, which have been proposed to mediate apical transport in polarized epithelial cells.

In polarized epithelial cells, and in the absence of a basolateral targeting signal, retroviral Env proteins are delivered to and virus buds from both membranes with equal efficiency, arguing that interactions with Env guide Gag to the basolateral surface.[137] The rhabdoviruses differ in this regard, in that even when the basolateral targeting signal for G has been mutated such that G is transported to the apical surface, virus assembly still occurs predominantly at the basolateral surface. Similarly, under conditions where the influenza virus HA is directed to the basolateral surface, virus budding remains predominantly apical. In these viruses, therefore, it appears that the internal components of the virion possess independent targeting signals that direct them to their respective membranes.[176]

Infection of lymphocytes by HIV-1 and MuLV results in polarized virus assembly at the uropod where virologic synapses are formed and result in efficient cell-cell spread.[62,113] Polarized virus assembly and release may, therefore, be important in determining the pathogenesis of viral infections since it can influence, in a major fashion, the pattern of virus spread in the infected host.[45]

Complex Interactions With the Secretory Pathway

While a majority of enveloped viruses undergo assembly and envelopment at a single site within the secretory pathway, some viruses have a more complex interaction that can involve de-envelopment or re-envelopment as part of their assembly pathway.

Rotavirus Assembly Within the ER

Rotaviruses are nonenveloped viruses that undergo transient envelopment at cellular membranes as an essential step in the formation of the mature double-shelled (triple layered) virus that is retained within the lumen of the ER until cell lysis. As with the reoviruses, the nucleocapsid, in this case containing 11 double-stranded RNA segments, is assembled in electron-dense cytoplasmic inclusions located close to the ER membranes, known as viroplasms. This assembly domain is dependent on expression of two nonstructural proteins, NSP2 and NSP5, and mutations or silencing of either gene product results in its abrogation.[73,139,202] In the viroplasm, genome replication occurs within early assembly structures that mature through the addition of multiple viral protein layers.[198] Following dsRNA synthesis, VP6, the most abundant protein of the virus, surrounds the inner core to form double-layered nucleocapsids with an outer icosahedral shell. They appear to bud directly into adjacent membranes that contain the two outer shell proteins VP7 and VP4. A nonstructural protein NSP4, which forms hetero-oligomers with the two outer shell proteins, mediates the interaction of the immature particle with the ER membrane.[162,189,264]

The exact topology of VP7 in the ER is not known. The mature protein has a cleaved signal peptide but is retained in the ER as if it were an integral membrane protein.[119,254] VP4 is thought to associate with VP7 and NSP4 just prior to budding of the nucleocapsid into the ER. Enveloped particles can be observed in the lumen of the ER, which then undergo a process of calcium-dependent de-envelopment, forming a triple layer particle an outer icosahedral shell of VP7 and VP4.[208] This process seems to be directed by VP7 since the silencing of this protein did not block the budding of double-layered particles into the ER but arrested maturation at the membrane-enveloped particle stage in this compartment.[227] The rapid association of VP4 with lipid rafts and the nonlytic release of rotaviruses from the apical plasma membrane of gut epithelial cells has led to the suggestion that the budding membranes surrounding the viroplasm might be ERGIC derived and that lipid rafts may play a role in transport of virus directly from this region to the PM.[50,60] Thus, this nonenveloped icosahedral virus appears to transiently utilize membranes of the secretory pathway as a scaffold on which to assemble an icosahedral shell and a nontraditional vesicle-mediated route to the PM.

Herpesvirus Envelopment in the Cytoplasm

As we described above, herpesvirus capsids that are assembled in the nucleus undergo a complex series of envelopment and de-envelopment steps termed “nuclear egress” in order to release the capsids into the cytoplasm (see: Assembly of Enveloped Viruses in the Nucleus and Fig. 6.11). It is here that tegument proteins of the mature virus, a collection of proteins including transcription factors located between the viral capsid and its lipid bilayer in the mature particle, are proposed to associate with the capsid prior to its re-envelopment by specialized vesicles that appear to be derived from multiple organelles, including the trans-Golgi network and early endosomes.[6,142] For herpes simplex virus and pseudorabies virus, glycoproteins of the gE/I complex together with gM appear to be essential for the re-envelopment step, and in their absence, intracytoplasmic aggregates of tegument-associated capsids are formed.[27]

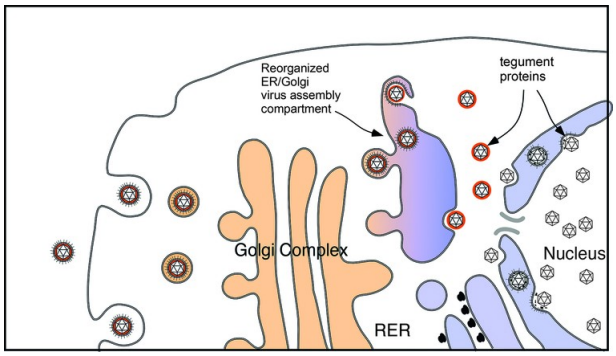

FIGURE 6.11 **Herpesvirus assembly**. In the re-envelopment pathway, nascent assembled capsids bud into the lumen of the nuclear membrane utilizing a subset of virally encoded glycoproteins. These enveloped particles then fuse with and are released from the outer nuclear membrane into the cytoplasm. There they associate with the tegument proteins and are transported through the cytoplasm to be enveloped by reorganized ER-Golgi–derived membranes that contain the full complement of virion glycoproteins.

Some of the clearest evidence for this complex assembly pathway comes from studies of HCMV and human herpesvirus 6 (HHV-6). In these viruses, the tegument is a dense structure that can be observed in electron micrographs. Enveloped virions in the lumen of the nuclear membrane display no evidence of tegument but acquire this layer following loss of the nuclear-derived envelope and subsequent release into the cytoplasm.[223,268] Studies of the glycoproteins of the gamma herpesvirus, Epstein-Barr virus, also support this model. Distribution of the viral glycoproteins gp110 and gp350/220 is consistent with the former being incorporated in virions at the nuclear membrane then lost on budding into the cytoplasm, while the latter is acquired only during re-envelopment in the cytoplasmic vesicles.[89] Similarly, studies of the lipid composition of extracellular HSV-1 virions indicate that it is similar to that of the Golgi complex and distinct from that of the nucleus.[273] Why would viruses evolve to utilize such a complex assembly strategy? Perhaps the most persuasive argument comes from the perspective of the neurotropic alphaherpesviruses. These viruses infect nerve endings in the periphery, but DNA replication and capsid assembly must occur in the cell body. In order to initiate a second round of infection at the nerve synapse, viral components must be transported down the axon. Thus, by separately transporting the viral capsids on microtubules and viral glycoproteins by the vesicular pathway, the site at which infectious virus is assembled can be much more accurately controlled.[159,267]

Poxvirus Acquisition of Multiple Membranes

Poxviruses, exemplified by vaccinia virus, exhibit an equally complex interaction with the secretory pathway. These large DNA viruses, which encode all of the machinery necessary for genomic replication and transcription, propagate entirely in the cytoplasm in specialized areas designated virus factories (see Chapters 67 and 68). The assembly and envelopment of these viruses is particularly complex since they apparently can be enclosed by multiple lipid bilayers reviewed in Ref.[46,219] The first recognizable structure is a crescent-shaped membrane that appears to be comprised of a single lipid bilayer with an external protein lattice constituted of trimers of the D13 protein.[100,262] Initial electron microscopy studies suggested that the crescent membranes that wrap the immature virions (IVs) are formed *de novo* within the virus factories,[55] and additional high-resolution electron microscopy experiments have been interpreted as support for the conclusion that the crescents are formed from a single membrane.[102] Nevertheless, the origin, mode of formation, and composition of these crescent membranes remain controversial. A number of investigators have provided evidence that these envelopes are composed of two closely apposed membranes, which are derived from the intermediate compartment (IC) of the secretory pathway.[215,252] Colocalization of IC resident cellular proteins with several vaccinia virus membrane proteins (A17L, A14L, and A13L) known to be critical for the formation of IV membranes have supported this model.[127,232] However, subsequent studies have shown that transport of proteins from the ER to the IC and Golgi is not necessary for IV formation, and that there is a transport pathway from the ER to the assembling IV.[106] Moreover, epitope tagging of the L2 protein, an early poxvirus protein essential for crescent formation, showed that it was associated with the ER tubules throughout the cytoplasm and in some instances appeared to be continuous with the membrane crescents.[148] The D13 protein, which forms a honeycomb lattice on the outer surface of the IVs, is critical for crescent formation and appears to act as a scaffold for their formation through its interaction with the N-terminus of the membrane spanning A17 protein.[20] Interestingly, Rouiller and coworkers have reported that the virus core of the related African Swine Fever virus core is also wrapped by specialized regions of the ER.[226] More recently, 3D electron tomography analysis using mutants that pause assembly at intermediate steps has revealed transient connections between immature viruses and ER membranes, providing further evidence that crescent membranes are ER derived.[282]

The process by which the nucleoprotein/transcription machinery is targeted into the immature envelopes is unclear, but a multiprotein complex of seven viral proteins, A15, A30, D2, D3, F10, G7, and J1, appears to be critical. Repression of several of these viral products yields the same phenotype; the failure of viral membranes and viroplasm to associate with each other.[261] Maturation of the IV to the infectious intracellular mature virus (IMV) involves a series of proteolytic cleavages of vaccinia structural proteins.[46] The mature virus particles are transported out of the assembly areas toward the periphery of the cell, where additional membranes that are derived from the trans-Golgi or early endosomal compartments wrap them to form the intracellular enveloped virus (IEV).[249,250] The membranes of these compartments contain vaccinia proteins that will be present in the external enveloped virus (EEV). Wrapping requires the participation of at least one protein present on the IMV and two EEV membrane proteins. It thus appears to be driven by interactions between vaccinia-encoded membrane proteins.[22,221] It is likely then that the IEV contains at least three concentric membranes, one derived from the ER and two from the Golgi/endosome. These particles are then transported via a reorganized actin cytoskeleton (so called actin-tails) to the plasma membrane,[51] where membrane fusion releases the infectious EEV form of the virus.

Modification of the Secretory Pathway

Transcription and Assembly of Poliovirus on a Disassembled Secretory Pathway

Poliovirus is a nonenveloped positive sense RNA virus that modifies the host cell extensively during its replication. Once introduced into a target cell, the genomic RNA is translated into a long polyprotein precursor that contains both structural and replicative proteins of the virus (Fig. 6.12). Early in the replication of the virus, there is a massive rearrangement of the intracellular membranes into clusters of vesicles 200-400 nm in diameter.[233] The altered membranes appear to have characteristics of autophagosomal vesicles since they have two membranes and contain, in addition to the viral RNA replication machinery, a number of autophagosomal proteins including LAMP1 and LC3. Moreover, stimulation of autophagy increased poliovirus yield, and inhibition of the autophagosomal pathway decreased virus yield, suggesting subversion of this pathway by poliovirus.[109,265] Electron microscopy studies suggested that the induced vesicles are derived from the host cell secretory pathway, since they appear to be connected to ER membranes early in infection and to the trans-Golgi network at later stages of infection.[155] These observations are supported by inhibitor studies demonstrating virus-induced vesicle formation and poliovirus replication are blocked by brefeldin A.[107] Viral RNA transcription occurs in close association with these vesicles, and both viral RNA and viral proteins known to be required for RNA replication have been shown by electron microscopy to be associated with their cytoplasmic surface.[18,269] Similarly, priming of RNA synthesis by the protein primer, VPg, appears to require membranes.[263] For poliovirus, the viral precursor protein 2BC when expressed alone was shown to cause both membrane vesiculation and the formation of multilamellar structures,[38] but it is in combination with poliovirus protein 3A, which causes the inhibition of ER to Golgi protein traffic,[66] that membrane alterations most consistent with the pattern in poliovirus-infected cells are observed.[259]

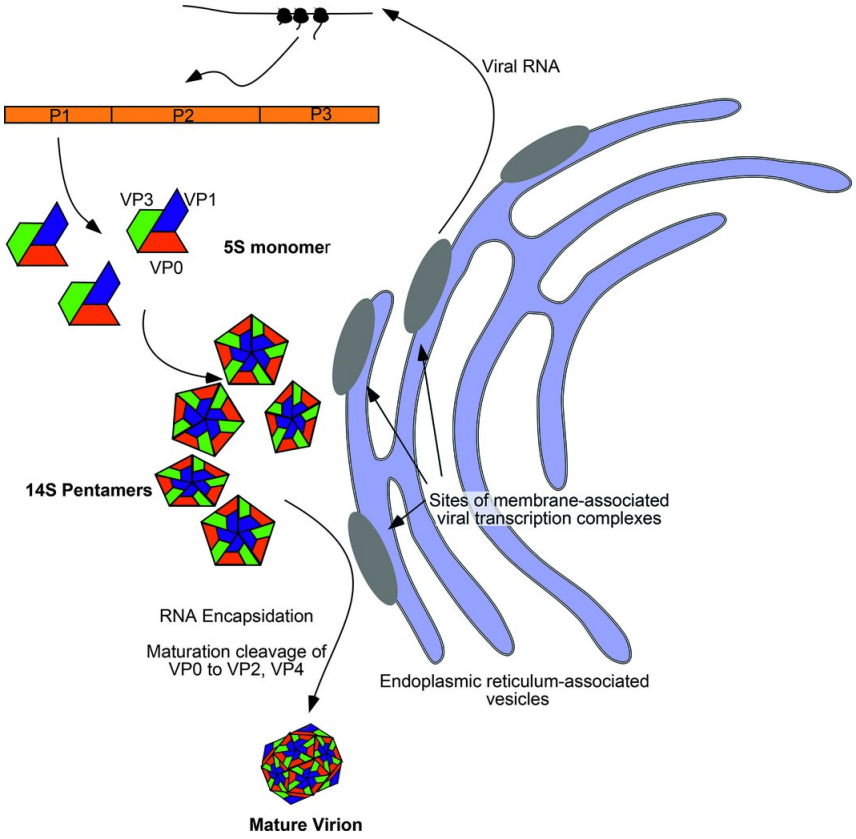

FIGURE 6.12 **Poliovirus assembly**. The P1 precursor protein is cleaved via an autocatalytic event from the nascent polyprotein and is then cleaved twice by the 3CD-protease to yield VP1, VP2, and VP0. This cleavage is essential for assembly of the 5S monomers into 14S pentamers. It is likely that 12 of these pentamers associate directly with nascent genomic RNA that is being transcribed from membrane-bound transcriptional complexes to assemble an immature 150S provirion. Maturation cleavage of VP0 to yield VP2 and VP4 results in conversion of these immature provirions into the 160S mature virion.

The formation of vesicular platforms for transcription may also be necessary for efficient capsid assembly. The poliovirus capsid appears to be assembled in a sequential process in which the 5S protomers containing VP1, VP3, and VP0, assemble to form 14S pentamers, which in turn are assembled into virus capsids (Fig. 6.12). Pentamers may associate with newly synthesized genomic RNA on intracytoplasmic vesicles and then assemble to form RNA-containing capsids upon completion of the RNA. This assembly pathway is supported by immunoelectron microscopy studies showing that pentamers associated with the replication complex can, if released by detergent, rapidly assemble into empty capsids —arguing that the vesicle prevents assembly of the capsid and keeps the pentamer in an appropriate configuration for association with the RNA.[205] In this instance, poliovirus appears to have circumvented the problems of intracytoplasmic localization of replicative and structural components by utilizing a membranous organizing center. Interestingly, mature polio virions can be found within vesicles that have been secreted from infected cells indicating that virus particles can form on the interior of virus-induced vesicles, which are then secreted as quasi-enveloped species.

Modification of Post-Golgi Vesicle pH by the Influenza Virus M2 Protein

The influenza hemagglutinin is synthesized as a precursor protein (HA0) that in the case of the avian influenza viruses is cleaved in the trans-Golgi by furinlike proteinases to the biologically functional HA1-HA2 heterodimer, three of which in turn form the trimeric HA complex (see Chapters 3 and 42). The influenza virus HA present on an infecting virion is activated to its fusogenic state by the low pH of the endosome. Thus, the similarly low pH of the trans-Golgi network and post-Golgi vesicles poses a problem for the virus during the assembly phase of its life cycle, if it is to maintain HA in a functional prefusogenic form. This is apparently solved by the action of the M2 protein, a small tetrameric protein

that forms cation-selective ion channels that can reduce the pH of the trans-Golgi network by pumping out hydrogen ions.[207,258]

Incorporation of the Nucleic Acid Genome During the Assembly Process

While the processes involved in the assembly of structural components of a virus are complex and interplay intimately with those of the host cell, they merely provide a mechanism by which the genomic information of the virus can be packaged into a protective environment for transfer to additional host cells. As such, the assembling virus must have evolved to efficiently select its genomic nucleic acid out of the pools of RNA and/or DNA that are present at the assembly site. Packaging of viral genomes generally involves a cis-acting sequence in the nucleic acid—the so-called packaging sequence—and a structural component(s) of the virus that can recognize and bind this element.

DNA Viruses

As described earlier, for adenovirus, an empty procapsid is first formed around scaffolding proteins, in a manner similar to that observed with DNA phages. Following procapsid assembly, DNA and associated core proteins are then subsequently packaged into these empty shells to yield "immature virions" that then undergo proteolytic maturation.

Early studies indicated that "light" virus particles contain subgenomic length viral DNA with an over-representation of left-end sequences. Subsequent studies showed that a cis-acting packaging sequence that is absolutely required for encapsidation of viral DNA is located within the left 390 bp of the genome. This region is highly conserved between different adenovirus subtypes and, in Ad 5, overlaps with two distinct enhancer elements for E1A transcription. Deletion of the packaging domain abrogates viability, but infectivity can be restored by substitution of the left-end sequences at the right end of the genome.[95,97] The packaging sequence only functions if it is within 600 bp of the inverted terminal repeat (ITS) but is not dependent on the ITS itself.[195] Mutational analyses showed that this packaging region contains at least seven functional AT-rich units called A-repeats. These repeats are functionally redundant but not functionally equivalent. Moreover, a sequence homology shared by the most important A repeats, 5′-TTTG-N_8-CG-3′, is essential for packaging domain function. Overexpression of an A-repeat multimer competes in *trans* resulting in a dramatic decrease in viral yield without affecting DNA replication or late-transcription, suggesting the involvement of limiting viral protein components in packaging.[91,238] It seems likely from a variety of genetic analyses, *in vitro* DNA binding studies, and chromatin immunoprecipitation approaches that the viral IVa2 protein together with the L4-22K protein bind directly to the A repeat sequences to initiate packaging.[72,194,196] The L1 52/55K protein, which is also critical for DNA packaging, is a part of this complex perhaps through interactions with IVa2 or L4-22K.[196,204]

Packaging of the herpesvirus genome is equally complex. As with the large DNA phages, herpesvirus DNA replicates to yield concatamers of genomes in a head to tail arrangement, and so packaging must be linked to the generation of genomic length units of DNA. In this virus, two packaging sequences, *pac1* and *pac2*, located within the terminal *a* repeats appear to be important for both recognition and cleavage of the DNA.[59] As with the DNA phages, a combination of cleavage recognition sequences and "head-full" packaging appears to ensure the incorporation of unit-length genomes into the procapsid. Studies utilizing a temperature-sensitive protease mutant of HSV-1, which accumulates procapsids (containing an uncleaved scaffolding protein) at the nonpermissive temperature, but which can remove the scaffold and package DNA upon temperature shift to the permissive temperature, have shown that ATP is necessary for DNA packaging to occur but DNA synthesis is not.[41,57] At least seven viral proteins, products of the genes UL6, UL15, UL17, UL25, UL28, UL32, and UL33, are required for cleavage and packaging of HSV-1 DNA. When cells are infected with HSV-1 mutants lacking the function of any of the seven genes, capsid formation, and DNA replication occur normally, but no packaging takes place. By analogy with the DNA bacteriophages such as P22, the portal vertex, assembled from 12 copies of the UL6 gene product, likely forms the docking site for these packaging proteins. Sequence analysis reveals that another critical component, encoded by the UL15 gene, shares homology with gp17, the large catalytic subunit of the bacteriophage T4 terminase. Thus, the product of this gene, together with that of UL28, which can specifically recognize the *pac* site, may play a direct role in the cleavage of viral DNA replication intermediates into monomers.[2,291]

RNA Viruses

For retroviruses, genomic packaging represents a particularly difficult problem since the assembling capsid must select genomic RNAs from a large pool of viral and cellular mRNAs that are also present in the cytoplasm. Viral RNAs to be specifically packaged are identified by the presence of an RNA sequence named the packaging signal or psi (ψ) and are selected from the cytoplasmic pool by a specific interaction with the zinc knuckle motif(s) of the NC domain of the Gag precursor.[54,140] The cis-acting RNA element is located at the 5′-end of the viral genome, has the capacity to mediate the packaging of heterologous RNAs, and is comprised of a series of stem-loop structures that each contribute to the strength and specificity of the signal. One of the best characterized, although perhaps most complex, ψ-sequence is that of HIV-1, which is now known to span the 5′ untranslated region of the genome, as well as downstream nucleotides in the Gag region for optimal packaging efficiency. This 5′ untranslated region, which can confer packaging specificity on foreign RNAs, includes the TAR sequence that is important in Tat-mediated transcriptional control, the adjacent poly-A loop, and four stem loops that incorporate the primer binding site (PBS), the dimerization sequence (DIS), the 5′-splice donor (SD), and the packaging signal (ψ), respectively. Gag has been shown to bind specifically to this region, with PBS, DIS, and ψ stem loops each providing independent high affinity binding sites. Disruptive and compensatory mutations within the TAR, Poly(A), PBS, DIS, and ψ elements indicate that the structure of the stem loops is important for packaging.[44,151] NMR analyses of SL3 in the presence and absence of the NC protein have shown that tight binding is mediated by specific interactions between the amino- and carboxyl-terminal CCHC-type zinc knuckles of the NC protein and nucleotide bases of the RNA loop.[58,140,295] In the context of the Gag precursor protein, however, an internal loop and the lower part of SL1 was discovered to be the major binding site on genomic RNA.[1]

Regions of retroviral genomes that promote genome packaging often overlap with segments that promote the RNA dimerization, which is necessary for packaging of the diploid genome. Moreover, there is mounting evidence that dimerization and packaging events are intimately coupled. An elegant structural analysis by D'Souza and Summers showed that dimerization of the MuLV genome results in an altered secondary structure in the psi region of the RNA, which exposes conserved UCUG elements that bind NC with high affinity. These elements are base paired and do not bind NC in the monomeric structure.[53] Thus, for MuLV, the problem of ensuring the encapsidation of two genomic RNAs has been solved by linking exposure of the packaging recognition sequence to dimer formation itself. For HIV-1, genomic RNA destined for dimerization and packaging is distinguished from those targeted for translation by the number of 5′ terminal G residues present. RNAs with a single G sequester the 5′ cap dimerize and can be packaged, whereas in those with 2 or 3 5′ G residues, the cap is accessible and recognized by the

translation machinery.[30]

The cellular location in which Gag:RNA recognition occurs remains an unanswered question. Parent et al. have provided strong evidence that for Rous sarcoma virus, genome packaging is dependent on Gag trafficking through the nucleus, a process that is mediated by NLS and NES signals in the MA domain of Gag.[80,94,200] However, the role of nuclear trafficking of HIV-1 Gag in RNA packaging remains unresolved and controversial.[140,200] Recent experiments by Hu and colleagues suggest that HIV-1 RNAs form dimers preferentially in the cytoplasm and that dimerization, mediated by the DIS sequence in the genome, is critical to efficient packaging into virions.[165,166] Live cell imaging of the incorporation of genomic RNA into assembling virions showed that in the absence of Gag, genomic RNA molecules were highly dynamic and did not localize at specific sites at or near the plasma membrane (PM). In contrast, in the presence of Gag, genomes were targeted to specific sites on the PM, where after initially exhibiting slow lateral movement they became fixed, and were the site for accumulation of additional Gag molecules.[115,117]

Genomic RNA packaging signals in other RNA viruses are less well defined. In the alphaviruses, this appears to involve direct binding of genomic RNA by the capsid protein. A short (132 nt) segment of RNA in the region of the genome encoding the nonstructural proteins, nsP1 or nsP2 depending on the alphavirus species, is critical for this interaction and for efficient packaging of genomic RNA. Like the retroviral psi-sequence, this packaging sequence (PS) has also been predicted to form a series of stem-loop structures.[122] The N-terminal third of the capsid protein is basic and is unstructured in the crystal structure; however, the balance of evidence suggests that the PS interacts strongly with a conserved sequence between residues 81 and 114 of the capsid protein.[156] While this sequence in the nsP coding region greatly enhances packaging, viral genomes without this sequence can still be packaged, albeit with low efficiently, through nonspecific interactions between RNA and capsid. This indicates that both specific and nonspecific interactions between viral genomes and capsid proteins are important for assembly events, but the genome packaging sequence increases the specificity of this process for viral genomes. Similarly, MHV genomes contains a packaging signal sequence that also increases the specificity for packaging of full viral genomes over the more abundant subgenomic RNAs but is not necessary for packaging.[149] For other positive-sense RNA viruses, multiple low-affinity capsid-binding signals found throughout the viral genome appear drive nucleocapsid assembly. For the bacteriophage MS2, numerous low-affinity RNA-capsid interactions facilitate a two-step process were RNA is first partially condensed, which then allows for higher order interactions that lead to encapsidation.[24] For hepatitis C virus, numerous low-affinity packaging signals are also found throughout the genome, which are thought to act synergistically in a mechanism that would parallel that of the MS2 bacteriophage.[253] Since assembled capsids have not been visualized in the cytosol of infected cells, it is thought that the final nucleation step occurs simultaneously with envelopment, but the precise mechanisms remain unclear.

For segmented viruses, such as the orthomyxoviruses or the reoviruses, genomic packaging requires that a complete set of segments be packaged together in a single virus particle. For influenza virus, reverse genetic approaches have shown that the signals for transcription and replication of an RNA segment are located in the 22 5′-terminal and the 26 3′ terminal nucleotides of the RNA.[141] These studies do not, however, address how a full complement of RNAs is selected during assembly. Two models have been proposed to accomplish this. The random incorporation model assumes a common structural feature in all the vRNPs, which enables them to be incorporated randomly into virions. Support for this model comes from the observation that an influenza A virion can possess more than eight vRNPs.[12,70] The selective-incorporation model predicts the presence of specific structures in each vRNA segment, leading to their individual incorporation into virions. Numerous lines of evidence strongly support a selective model for genome packaging.[185] First is data showing that defective RNA segments could inhibit the packaging of the parental segment into virions.[69] Second was the finding that viruses grown in the presence of bacterial neuraminidase and antibody to NA retained an extensively deleted NA vRNA, suggesting that the altered NA segment participates in viral replication and carries structural features required for its incorporation into virions.[289] Experiments utilizing reverse genetics approaches have further supported the selective incorporation model by demonstrating that all eight vRNA segments must be present for efficient virion formation and that sequences within the coding region of the NA vRNA encode a signal that drives incorporation of this segment into virions. Subsequently, genome packaging signals unique to each of the other seven viral RNA segments have been identified.[79,132,185] Electron microscopic studies of serially sectioned influenza virus A particles, which showed the RNPs organized in a distinct pattern (seven segments of different lengths surrounding a central segment), also support a model where the eight segments are cooperatively packaged into virions through intersegment associations[185] (Fig. 6.13).

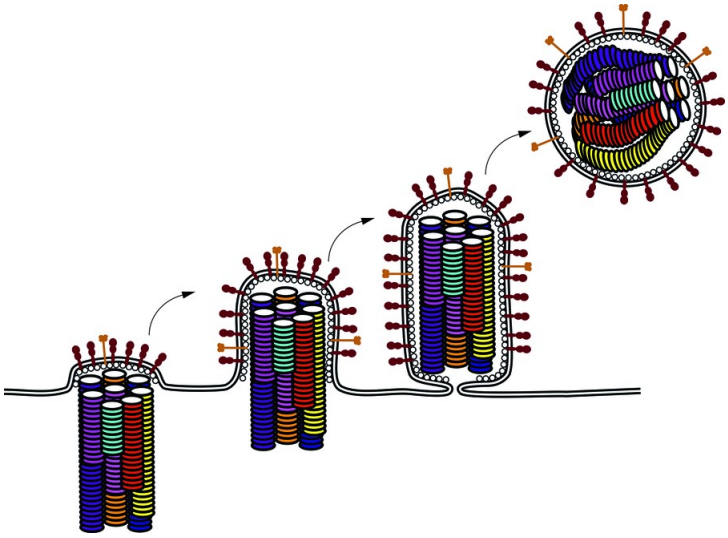

FIGURE 6.13 **Influenza virus budding**. Incorporation of influenza virus genome segments into budding virions at the plasma membrane involves specific packaging sequences found in each genome segment ribonucleoprotein (RNP) complex. These RNP complexes assemble at the plasma membrane in a 7 + 1 configuration through interactions with viral glycoproteins HA and NA. Both glycoproteins and the M1 protein contribute to bud formation and virion shape. Scission and release of the virus particle is facilitated by

viral M2, which localizes to the neck of the budding virion. (Modified from Noda T, Kawaoka Y. Structure of influenza virus ribonucleoprotein complexes and their packaging into virions. *Rev Med Virol.* 2010;20(6):380-391. Copyright © 2010 John Wiley & Sons, Ltd. Reprinted by permission of John Wiley & Sons, Inc. Ref.[186])

Postassembly Modifications and Virus Release

Proteolytic Cleavage and Virus Maturation

Proteolytic enzymes play important roles in the process of assembly for many viruses and in a postassembly maturation step that is required for the development of an infectious particle for others. These cleavage events can act as molecular switches that introduce flexibility as well as irreversibility into the assembly process. Additionally, maturation events can function to limit reinfection or glycoprotein fusion events before newly assembled virus has exited the cell.

Proteolytic cleavage in the case of the alphaviruses, for example, is used to allow protein domains, translated from a single mRNA, to enter different transport pathways in the cell. The C protein, which is encoded at the 5′ end of the subgenomic 26S mRNA, folds cotranslationally into an active serine proteinase that cleaves itself from the growing polypeptide chain. This releases the C protein into the cytoplasm for capsid assembly while freeing up the N-terminus of the signal peptide for the p62 glycoprotein precursor, which can then bind SRP and direct the ribosome to the ER.[82]

Numerous proteolytic cleavage events are required for flavivirus particle formation and production of infectious particles.[14,184] Dengue virus C proteins are initially synthesized with a 3′ transmembrane domain, which anchors it in the membrane and connects it to the luminal prM glycoprotein. Proteolytic cleavage of this domain by signal peptidase on the luminal side and the viral serine protease NS2B/3 on the cytosolic side initiates budding by allowing the glycoproteins to form oligomer structures and the C protein to initiate nucleocapsid formation. Enveloped flavivirus particles form by budding into the ER in an immature form that contains the precursor prM glycoprotein on its surface. During transit of these immature viral particles to the plasma membrane through the secretory pathway, the prM glycoprotein is cleaved by the host protease furin to form pr and M. Once secreted from the cell, the pr domain is released from the viral particle, freeing the fusion peptide to form infectious particles.

In the herpesviruses, proteolytic cleavage of the scaffolding protein occurs after assembly of the procapsid is complete and is a prerequisite for DNA packaging. The scaffolding proteins of HSV-1 are encoded by a pair of overlapping genes, UL26 and UL26.5, in which the open reading frame of UL26 is an in-frame N-terminal extension of that of UL26.5. The product of the smaller gene is the abundant scaffolding protein pre-VP22a, while the larger gene encodes a protease precursor, which cleaves itself internally to give the proteins VP24 (protease) and VP21. Temperature-sensitive mutants in UL26 that have an inactive proteinase at the nonpermissive temperature are blocked at the procapsid stage of assembly reviewed in Ref.[216]

A somewhat more complex proteolytic pathway is utilized by the picornaviruses to regulate the release of both structural and replicative protein components from the single polyprotein precursor that is translated from its positive-stranded RNA genome (Fig. 6.12). The initial event in this proteolytic cascade is the primary cleavage in which the P1 structural protein precursor is separated from the P2-P3 precursor. This reaction is catalyzed by the 2A proteinase, which hydrolyzes a Tyr-Gly bond at its own amino-terminus. While cleavage of the P2-P3 region is carried out by the 3C proteinase at Gln-Gly bonds, the efficient cleavage of the structural protein precursor at the VP0-VP3 and VP3-VP1 boundaries is catalyzed specifically by the 3CD precursor that includes the viral polymerase. Cleavage of the P1 precursor appears to be a prerequisite for entry of the capsid proteins into the assembly pathway. The final processing step is cleavage of VP0-VP4 and VP2. This autocatalytic, maturational cleavage occurs late in the assembly pathway and appears to be linked to encapsidation of the viral RNA genome. It is likely that the structural alterations that accompany the cleavage event both stabilize the capsid and irreversibly commit the virus to the conformational changes induced on receptor binding that are necessary for productive infection of cells.[99]

A similar maturational cleavage is required following assembly and envelopment of retroviral capsids. As we have described earlier, the retroviral capsid is assembled from polyprotein precursors that are encoded by the *gag* gene. The viral aspartyl proteinase is encoded by the *pro* region of the genome and is translated from unspliced, genome length RNA by a ribosomal frameshift mechanism as a Gag-Pro or Gag-Pro-Pol precursor protein (see Chapters 48 and 50). The presence of the Gag sequences ensures that this key enzyme is targeted to and incorporated into the assembling capsid. The mechanism by which the protease is activated late in the assembly process (most probably after envelopment is complete) is not known. Since the active enzyme is a homodimer of two protease subunits, it has been postulated that dimerization of proteinase precursors in the nascent capsid might allow a functional enzyme to form. However, in the betaretroviruses, the immature capsid contains a full complement of proteinase precursors, which are not activated until late in envelopment at the plasma membrane. Immature capsids of the betaretrovirus M-PMV are stable when isolated from infected cells, but the protease can be activated to cleave the assembled precursors to their mature products by treatment with the reducing agent dithiothreitol, suggesting that modification of cysteine residues might act to regulate the initiation of proteolysis.[201]

Cleavage of the Gag precursors in the immature capsid is accompanied by a major morphological rearrangement in which the mature NC protein condenses the genomic RNA inside a CA shell (Fig. 6.7)—the electron-dense core structure of the virus seen in electron micrographs.[85] Cleavage at the N-terminus of CA induces a conformational change in the protein that allows an N-terminal β-hairpin loop to form, while cleavage at the C-terminus removes the CA-SP1 six-helix bundle. This disassembly and structural rearrangement of CA redirects assembly from a “spherical” immature capsid to that of the conelike core-shell structure formed by CA hexamers and pentamers.[130,150] Maturational cleavage, as in the case of poliovirus, results in an irreversible commitment to the entry pathway. It prevents a simple reversal of the assembly process and presumably through the formation of the core allows the rearrangement of RNA and the reverse transcriptase into a conformation that is optimal for reverse transcription of the genome. In the absence of cleavage, reverse transcription cannot occur and the virions are noninfectious.[260]

Adenoviruses, like retroviruses, undergo a maturational cleavage after assembly that is essential for infectivity. The immature virions formed after DNA packaging contain five precursor proteins that must be processed before the mature infectious virion is produced. These include components of the core and the preterminal protein (pTP) used to initiate DNA synthesis. The 23-kD adenovirus proteinase, which is encoded by the L3 region of the genome, is inactive in its purified form. It requires an 11 amino acid peptide present at the carboxy-terminus of pVI and viral DNA as cofactors for its activity. This unique use of DNA as a cofactor ensures that proteolytic maturation of the virus cannot take place

until packaging of DNA is complete.[52,145]

Nonproteolytic Maturation Events

Hepatitis B virus particles undergo several postassembly modifications to form infectious particles but, unlike many other virus maturation events, these do not rely of proteolytic cleavage. HBV capsids form around viral RNA, which is then reverse transcribed and replicated to form mature capsids containing the dsDNA genome.[242] The synthesis of dsDNA alters the capsid structure, directing it for envelopment and secretion. Once outside the cell, the HBV envelope protein S antigen undergoes a time-dependent morphological change, altering their membrane orientation to expose the receptor-binding domain.[241] Mature HBV particles bind to heparan sulfate proteoglycans, which are abundant in many tissues. Therefore, this maturation strategy ensures that HBV virions can circulate in the blood long enough to arrive in the liver without being sequestered. Once in the liver, specific environmental conditions seem to promote conversion from immature to mature particles resulting in liver-specific infection.

Role of Viral and Cellular Proteins in Membrane Extrusion

It is clear from the discussions in previous sections that different strategies are utilized by different enveloped viruses to mediate the process of membrane extrusion that we have termed budding. Envelopment by a lipid bilayer fulfills two functions for a virus: it provides a protective outer layer into which can be embedded the necessary machinery for target cell attachment and entry, and, in most cases, it releases the virus into an environment that is topologically equivalent to the exterior of the cell. The strategies used to drive the budding process can be classified into three general mechanisms.[81] In the first, membrane extrusion is driven by envelope glycoproteins alone. This is exemplified by the E protein of the coronaviruses that can induce the release of VLPs in the absence of an RNP. The second strategy is that used by the retroviruses where the capsid precursors, in the absence of the viral glycoproteins, can efficiently induce membrane extrusion and particle release. In this instance, it is hypothesized that the tight interaction between the MA domain of Gag and the lipid bilayer coupled with the force of Gag-Gag interactions in assembly drives the budding process. The third mechanism requires both the glycoproteins and capsid to drive budding. This strategy is used by alphaviruses, which involves interactions between the viral glycoprotein spikes and the assembled capsid, as well as *Flaviviridae* viruses where capsid assembly and budding occur simultaneously. It appears that lateral interactions between the glycoproteins, coupled with cytoplasmic domain-capsid connections, progressively bend the membrane to form the spherical particle.[82]

For all of these mechanisms, the final step of budding, the process of pinching off, requires a membrane fission event. For several viruses, it is now clear that this is a separable event that requires the involvement of both viral and host components. Experiments in HIV showed that deletion of the p6 domain at the C-terminus of Gag results in a block to virus release at the pinching off stage,[90] and it is now understood that diverse retroviruses encode functionally equivalent Gag “late function” or L domains that are required for efficient release of virions.[61,169] Viral L domains fall into three classes based on their characteristic tetrapeptide sequence motifs, Pro-Thr/Ser-Ala-Pro (PTAP, or PSAP), Pro-Pro-X-Tyr (PPXY), and Tyr-Pro-$(X)_n$-Leu (YP$(X)_n$L). In many instances, these viral L domains are functionally interchangeable and can mediate pinching off of the virion even when positioned at different locations in Gag.[199,294] Evidence has accumulated over the past several years that these motifs interact with components of a complex cellular machinery that is used in the biogenesis of an endosomal compartment known as the multivesicular body (MVB). Unlike other mechanisms used by the cell to form and pinch off vesicles into the cytoplasm, this machinery facilitates the pinching off into the MVB of exosomes that have the same topology as virus. This process is mediated by a complex and highly conserved protein network, the ESCRT (endosomal sorting complex required for transport) machinery, which exists in all eukaryotes and consists of 5 hetero-oligomeric complexes (ESCRT-0, ESCRT-1, ESCRT-II, ESCRT-III, and VPS-4).[130,158,283] Components of this complex also appear to be involved in the last stage of cytokinesis, termed abscission, where, as with virus release, a membranous stalk must undergo membrane fission to release the two daughter cells.[32,167] Recent studies have shown that the ESCRT-0 complex functions to concentrate ubiquitinated cargo on endosomal membranes, the ESCRT-I and -II complexes together induce bud formation, while remaining outside the vesicle, and the ESCRT-III complex mediates membrane scission from the cytosolic side of the bud.[286] Mammalian ESCRT-III is formed by charged multivesicular body proteins (CHMPs), a family of structurally related but highly divergent α-helical proteins that has 12 known members in humans.[283]

The initial indication that retroviral budding and this machinery were linked came from the observation that the ESCRT-I component Tsg101 interacted with p6 and that this interaction was dependent on the PTAP motif.[83] A Tsg101 interacting protein, ALIX, which can also interact directly with ESCRT-III, has been shown to interact with the YP$(X)_n$L motif in both equine infectious anemia virus and HIV.[255] The PPXY motif, which was first identified in Rous sarcoma virus, is utilized by several viruses including other retroviruses, filoviruses, arenaviruses, rhabdoviruses, and hepadnaviruses.[158] It plays a key role in recruiting host WW domain–containing proteins of the Nedd4 family, ubiquitin E3 ligases involved in the covalent attachment of ubiquitin to membrane-associated proteins.[231,290] Mono-ubquitination has been shown to direct the modified protein to the MVB and likely functions to link PPXY L-domain proteins to the ESCRT machinery.[169]

In addition to those L-domains that play a major role in virus release, it is becoming clear that in several viruses multiple auxiliary L-domains are present that can augment the process. In HIV-1, for example, there is evidence that the PTAP motif acts synergistically with ALIX-binding sequences at the C-terminus of p6 to optimize release.[147] Through these disparate mechanisms,[147,283] viruses recruit components of ESCRT-III to the site of budding to mediate the process of membrane scission. Only a subset of the 12 proteins known to make up the ESCRT-III complex may be involved since a recent study demonstrated that the codepletion of CHMP2 or CHMP4 family members profoundly impairs HIV-1 release, whereas siRNA knock down of other CHMPs had limited impact.[168]

The ATPase VPS4 (vacuolar protein sorting 4) acts at a late stage of ESCRT function, providing energy for ESCRT dissociation and membrane scission. Live cell imaging studies reveal that ESCRT-III and VPS4 are rapidly and transiently recruited before HIV-1 particle release, supporting the notion that VPS4 has a direct role in this stage of HIV-1 budding.[15,118]

Influenza virus is an exception to this widespread dependence on the ESCRT machinery.[31] Rossman and colleagues showed that for this virus, it is the viral M2 protein, rather than a host component, that mediates virus membrane scission.[224] During morphogenesis, the M2 protein localizes to the neck of budding virions, and a highly conserved amphipathic helix in the cytoplasmic tail appears to mediate a cholesterol-dependent alteration in membrane curvature. Mutation of the amphipathic helix results in failure of the virus to undergo membrane scission

and virion release.[225] Thus, it is possible that viral proteins such as M2 are able to mimic the functions of the ESCRT III machinery by constricting the viral membrane and inducing the scission events required for virus release.

Mechanisms to Facilitate the Release of Nascent Particles

If they are to efficiently travel to an uninfected target cell, viruses face one last hurdle following envelopment and release. This is the need to avoid rebinding to receptors on the producing cell. For viruses such as the ortho- and paramyxoviruses, which utilize sialic acid as a receptor on the cell surface, this problem is circumvented by the incorporation of a receptor-destroying enzyme, a sialidase, which can remove these residues during transit through the secretory pathway. For influenza virus, the neuraminidase is critical for virus release since in its absence nascent virus binds to both the cell surface and other virions in large aggregates.[136]

Retroviruses have similarly evolved mechanisms to facilitate release and thereby reduce superinfection of a cell. Cells infected with one retrovirus are generally resistant to subsequent infection by a second virus of the same receptor class. The presumed mechanism for this viral interference is the synthesis of viral glycoproteins in large excess over the levels that are incorporated into virions with the effect that cellular receptors that are transported to the cell surface are already occupied by their ligand. This results in removal or downregulation of the receptor from the cell surface. For HIV-1 downregulation of the primary receptor, CD4 seems to be crucial since the virus has evolved two additional mechanisms to ensure this. Two of the accessory proteins encoded by HIV, Vpu and Nef, act by binding CD4 in the ER and at the cell surface, respectively, and targeting the receptor for degradation.

Independent of this need to downregulate viral receptors, recent work has demonstrated that virus escape from the cell surface, the last step of the replication cycle, is effectively targeted by a host-cell restriction factor, tetherin (also known as BST2 or CD317). This protein was initially identified as the factor responsible for the block to virion release from certain cell types infected by HIV-1 mutants lacking the accessory gene *vpu*.[17,147,178,271] Tetherin can be induced by type I interferon and other proinflammatory stimuli and has an unusual topology in which the extracellular domain forms an extended parallel coiled-coil that is anchored at the N-terminus by a protein transmembrane domain and at the C-terminus by a glycosyl phosphatidylinositol GPI anchor. Biochemical and structural data favor a model where parallel tetherin dimers cross-link virions to the plasma membrane or the membrane of other virions.[17,147] Tetherin can restrict the release of all retroviral particles tested to date, including those of the spumavirus and betaretrovirus families, which preassemble capsids in the cytoplasm. In addition, mammalian tetherins block release of a variety of membrane viruses including filoviruses, arenaviruses, rhabdoviruses, and at least one herpesvirus.[71,116] In turn, viruses have evolved countermeasures that have allowed them to overcome this inhibition of release. The Vpu protein of HIV-1 is the prototypical antagonist to tetherin and interacts through the transmembrane region to mediate downregulation and lysosomal degradation of the host restriction factor. In the case of SIVs, this function is primarily carried out by the Nef protein, although in some instances, as in HIV-2 or Ebola virus, it can be mediated by the viral glycoprotein.[71] Given the diversity of mechanisms utilized to counteract tetherin, it is clear that this last step of the life cycle is critical to the virus. Indeed, the evolution of primate lentiviruses, particularly pandemic HIV-1, has been shaped by species-specific differences in this host-cell restriction factor.[236]

Cell-Associated and Noncanonical Spread of Infection

In addition to cell-free spread of virus infection that involves release of virions from infected cells, many virus infections are spread through directed processes that limit extracellular exposure.[43] Cell-associated spread provides several advantages for virus infection including limiting neutralization by extracellular immune responses and rapid movement of multiple genomes between cells. This can occur through the secretion of fully formed virions that are specifically targeted to uninfected cells, through secretion of virions in cellular exosomes or through the direct transfer of genetic material between cells without the formation of virus particles.

Several mechanisms have been described for selective targeting of virions to uninfected. Retroviruses utilize the close contact between lymphocyte cells to spread directly between cells through a “virological synapse.” For human T-cell leukemia virus type 1, contact between lymphocytes stimulate a microtubule polarization of the cell, which leads to recruitment of Gag and Env proteins to the cell contact site. This causes a rapid secretion of virus particles that are quickly taken up by the uninfected cell. Virus particles from numerous different species have also been observed to travel between cells along cellular extrusions such as actin tails or filopodia. Once outside the cells, vaccinia virus particles are propelled on virus-induced actin tails toward new cells. Virus infection often increases the formation of cellular filopodia, and filopodia-associated virus particles have been observed in several virus infections including murine leukemia virus (MLV), alphaviruses, and coronaviruses. In the case of MLV, viral particles move along the outside of filopodia to adjacent cells where they are taken up to initiate infection. On the other hand, alphavirus particles move along the interior of filopodia and bud from the tip in close contact to adjacent cells. In each case, virus particles retain cellular contacts or are released in a restricted space that limits the availability for neutralization.

In an alternative strategy for virus spread, several virus infections have been reported to spread directly between cells without the formation of virus particles. These mechanisms rely on the movement of viral genomic material to new cells through cellular channels or on the fusion of infected cells with uninfected cells. Influenza virus proteins and genetic material appear to transit on tunneling nanotubes that normally form channels between adjacent cells to allow cytosolic exchange.[42] Traveling along these intracellular pathways facilitates influenza virus cell to cell spread and genome reassortment. Paralleling this mechanism, measles virus infection leads to the formation of membrane pores between adjacent cells through which viral genomes are directly transferred. Alternatively, virus infections can spread through tissues by fusion of infected cells with uninfected cells to form large multinucleated syncytia. Respiratory syncytia virus, HIV, herpes simplex virus, and coronavirus infections can all lead to cell fusion mediated by the viral envelope proteins.[131] For coronaviruses, the fraction of S protein that localizes to the plasma membrane can interact with cellular receptor proteins on adjacent cells leading to an activation of the spike fusion peptide and subsequent cellular membrane fusion. In SARS-CoV-2 infections, large multinucleated cells are often observed in severe Covid-19 cases leading to the prediction that syncytia formation is involved in viral pathogenesis; however, the precise function of syncytia formation in coronavirus spread or pathogenesis is still poorly understood.

Finally, recent evidence demonstrates that assembled virus particles can be transferred between cells in host-derived extracellular vesicles (EVs). Notably, virions from several nonenveloped viruses are often found in EVs forming quasi-enveloped virus particles that are nonlytically released from host cells.[75] Examples include poliovirus, coxsackievirus, hepatitis A virus (HAV), and hepatitis E virus (HEV).[74,172] For enteroviruses such as poliovirus and coxsackievirus, the formation of virus-containing EVs is linked to the autophagosome secretion pathway.[172] Poliovirus virions are found inside LC3-II positive autophagosome-like double membrane vesicles, which traffic from the cytosol to the plasma membrane

and where the outer membrane fuses with the plasma membrane to release a single membrane EV containing multiple virions.[35] These enterovirus-containing EVs are enriched in phosphatidylserine (PS) lipids, which are thought to act both as a receptor for uptake in uninfected cells and as potent anti-inflammatory molecules. Similar mechanisms of secretion and spread through EVs have been observed for other enteroviruses as well as rotavirus, norovirus, and Zika virus.[235,297] Contrasting enterovirus-containing EVs that are enriched for autophagosome machinery, HAV-containing EVs are smaller, contain fewer virus particles, and have a protein composition comparable to exosomes. HAV budding into EVs is mediated by interactions between the C-terminal pX peptide of the viral VP1 capsid protein and components of the ESCRT machinery.[245,280] Although the site of budding is still unknown, the enrichment of endosomal proteins in HAV EVs argues for intracellular budding into multivesicular bodies followed by release at the plasma membrane.[74] Within infected individuals, HAV-containing EVs are the primary virion form found circulating in the blood.

Similar to other strategies for cell-associated spread, virion-containing EVs can provide efficient transfer of multiple viral genomes to uninfected cells while shielding viral epitopes from neutralizing antibodies. For RNA viruses that often accumulate mutations during replication, infection with multiple virus particles is an important avenue to overcome limiting mutations within individual genomes and decreases the barrier to establishing a productive infection. EVs have also proven more stable in harsh extracellular environments such as the GI tract and can be more resistant disinfectants.[296]

REFERENCES

1. Abd El-Wahab EW, Smyth RP, Mailler E, et al. Specific recognition of the HIV-1 genomic RNA by the Gag precursor. Nat Commun. 2014;5:4304.

2. Adelman K, Salmon B, Baines JD. Herpes simplex virus DNA packaging sequences adopt novel structures that are specifically recognized by a component of the cleavage and packaging machinery. Proc Natl Acad Sci U S A. 2001;98(6):3086-3091.

3. Ahi YS, Mittal SK. Components of adenovirus genome packaging. Front Microbiol. 2016;7:1503.

4. Akarsu H, Burmeister WP, Petosa C, et al. Crystal structure of the M1 protein-binding domain of the influenza A virus nuclear export protein (NEP/NS2). EMBO J. 2003;22(18):4646-4655.

5. Alber F, Dokudovskaya S, Veenhoff LM, et al. The molecular architecture of the nuclear pore complex. Nature. 2007;450(7170):695-701.

6. Alwine JC. The human cytomegalovirus assembly compartment: a masterpiece of viral manipulation of cellular processes that facilitates assembly and egress. PLoS Pathog. 2012;8(9):e1002878.

7. Aridor M. A tango for coats and membranes: new insights into ER-to-Golgi traffic. Cell Rep. 2022;38(3):110258.

8. Avalos RT, Yu Z, Nayak DP. Association of influenza virus NP and M1 proteins with cellular cytoskeletal elements in influenza virus-infected cells. J Virol. 1997;71(4):2947-2958.

9. Baines JD. Herpes simplex virus capsid assembly and DNA packaging: a present and future antiviral drug target. Trends Microbiol. 2011;19(12):606-613.

10. Balasubramaniam M, Freed EO. New insights into HIV assembly and trafficking. Physiology (Bethesda). 2011;26(4):236-251.

11. Baldwin DN, Linial ML. The roles of Pol and Env in the assembly pathway of human foamy virus. J Virol. 1998;72(5):3658-3665.

12. Bancroft CT, Parslow TG. Evidence for segment-nonspecific packaging of the influenza a virus genome. J Virol. 2002;76(14):7133-7139.

13. Barlowe C, Helenius A. Cargo capture and bulk flow in the early secretory pathway. Annu Rev Cell Dev Biol. 2016;32:197-222.

14. Barnard TR, Abram QH, Lin QF, Wang AB, Sagan SM. Molecular determinants of flavivirus virion assembly. Trends Biochem Sci. 2021;46(5):378-390.

15. Baumgartel V, Ivanchenko S, Dupont A, et al. Live-cell visualization of dynamics of HIV budding site interactions with an ESCRT component. Nat Cell Biol. 2011;13(4):469-474.

16. Bhattacharya J, Repik A, Clapham PR. Gag regulates association of human immunodeficiency virus type 1 envelope with detergent-resistant membranes. J Virol. 2006;80(11):5292-5300.

17. Bieniasz PD. The cell biology of HIV-1 virion genesis. Cell Host Microbe. 2009;5(6):550-558.

18. Bienz K, Egger D, Pasamontes L. Association of polioviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualized by electron microscopic immunocytochemistry and autoradiography. Virology. 1987;160(1):220-226.

19. Billeter MA, Cattaneo R, Spielhofer P, et al. Generation and properties of measles virus mutations typically associated with subacute sclerosing panencephalitis. Ann N Y Acad Sci. 1994;724:367-377.

20. Bisht H, Weisberg AS, Szajner P, Moss B. Assembly and disassembly of the capsid-like external scaffold of immature virions during vaccinia virus morphogenesis. J Virol. 2009;83(18):9140-9150.

21. Blackburn JB, D'Souza Z, Lupashin VV. Maintaining order: COG complex controls Golgi trafficking, processing, and sorting. FEBS Lett. 2019;593(17):2466-2487.

22. Blasco R, Moss B. Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-Dalton outer envelope protein. J Virol. 1991;65(11):5910-5920.

23. Bonifacino JS, Glick BS. The mechanisms of vesicle budding and fusion. Cell. 2004;116(2):153-166.

24. Borodavka A, Tuma R, Stockley PG. Evidence that viral RNAs have evolved for efficient, two-stage packaging. Proc Natl Acad Sci U S A. 2012;109(39):15769-15774.

25. Boson B, Legros V, Zhou B, et al. The SARS-CoV-2 envelope and membrane proteins modulate maturation and retention of the spike protein, allowing assembly of virus-like particles. J Biol Chem. 2021;296:100111.

26. Bourmakina SV, Garcia-Sastre A. Reverse genetics studies on the filamentous morphology of influenza A virus. J Gen Virol. 2003;84(Pt 3):517-527.

27. Brack AR, Dijkstra JM, Granzow H, Klupp BG, Mettenleiter TC. Inhibition of virion maturation by simultaneous deletion of glycoproteins E, I, and M of pseudorabies virus. J Virol. 1999;73(7):5364-5372.

28. Briggs JA, Krausslich HG. The molecular architecture of HIV. J Mol Biol. 2011;410(4):491-500.

29. Brodsky JL, Skach WR. Protein folding and quality control in the endoplasmic reticulum: recent lessons from yeast and mammalian cell systems. Curr Opin Cell Biol. 2011;23(4):464-475.

30. Brown JD, Kharytonchyk S, Chaudry I, et al. Structural basis for transcriptional start site control of HIV-1 RNA fate. Science. 2020;368(6489):413-417.

31. Bruce EA, Medcalf L, Crump CM, et al. Budding of filamentous and non-filamentous influenza A virus occurs via a VPS4 and VPS28-independent pathway. Virology. 2009;390(2):268-278.

32. Carlton JG, Martin-Serrano J. Parallels between cytokinesis and retroviral budding: a role for the ESCRT machinery. Science. 2007;316(5833):1908-1912.

33. Cepko CL, Sharp PA. Assembly of adenovirus major capsid protein is mediated by a nonvirion protein. Cell. 1982;31(2 Pt 1):407-415.

34. Chazal N, Gerlier D. Virus entry, assembly, budding, and membrane rafts. Microbiol Mol Biol Rev. 2003;67(2):226-237.

35. Chen YH, Du W, Hagemeijer MC, et al. Phosphatidylserine vesicles enable efficient en bloc transmission of enteroviruses. Cell. 2015;160(4):619-630.

36. Cheng F, Mukhopadhyay S. Generating enveloped virus-like particles with in vitro assembled cores. Virology. 2011;413(2):153-160.

37. Chlanda P, Schraidt O, Kummer S, et al. Structural analysis of the roles of influenza A virus membrane-associated proteins in assembly and morphology. J Virol. 2015;89(17):8957-8966.

38. Cho MW, Teterina N, Egger D, Bienz K, Ehrenfeld E. Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells. Virology. 1994;202(1):129-145.

39. Choi G, Park S, Choi B, et al. Identification of a cytoplasmic targeting/retention signal in a retroviral Gag polyprotein. J Virol. 1999;73(7):5431-5437.

40. Christensen JB, Byrd SA, Walker AK, Strahler JR, Andrews PC, Imperiale MJ. Presence of the adenovirus IVa2 protein at a single vertex of the mature virion. J Virol. 2008;82(18):9086-9093.

41. Church GA, Dasgupta A, Wilson DW. Herpes simplex virus DNA packaging without measurable DNA synthesis. J Virol. 1998;72(4):2745-2751.

42. Cifuentes-Munoz N, Dutch RE, Cattaneo R. Direct cell-to-cell transmission of respiratory viruses: The fast lanes. PLoS Pathog. 2018;14(6):e1007015.

43. Cifuentes-Munoz N, El Najjar F, Dutch RE. Viral cell-to-cell spread: conventional and non-conventional ways. Adv Virus Res. 2020;108:85-125.

44. Clever JL, Eckstein DA, Parslow TG. Genetic dissociation of the encapsidation and reverse transcription functions in the 5′ R region of human immunodeficiency virus type 1. J Virol. 1999;73(1):101-109.

45. Compans RW. Virus entry and release in polarized epithelial cells. Curr Top Microbiol Immunol. 1995;202:209-219.

46. Condit RC, Moussatche N, Traktman P. In a nutshell: structure and assembly of the vaccinia virion. Adv Virus Res. 2006;66:31-124.

47. Corse E, Machamer CE. Infectious bronchitis virus E protein is targeted to the Golgi complex and directs release of virus-like particles. J Virol. 2000;74(9):4319-4326.

48. Cortese M, Goellner S, Acosta EG, et al. Ultrastructural characterization of zika virus replication factories. Cell Rep. 2017;18(9):2113-2123.

49. Craven RC, Parent LJ. Dynamic interactions of the Gag polyprotein. Curr Top Microbiol Immunol. 1996;214:65-94.

50. Cuadras MA, Greenberg HB. Rotavirus infectious particles use lipid rafts during replication for transport to the cell surface in vitro and in vivo. Virology. 2003;313(1):308-321.

51. Cudmore S, Reckmann I, Griffiths G, Way M. Vaccinia virus: a model system for actin-membrane interactions. J Cell Sci. 1996;109(Pt 7):1739-1747.

52. D'Halluin JC. Virus assembly. Curr Top Microbiol Immunol. 1995;199(Pt 1):47-66.

53. D'Souza V, Summers MF. Structural basis for packaging the dimeric genome of Moloney murine leukaemia virus. Nature. 2004;431(7008):586-590.

54. D'Souza V, Summers MF. How retroviruses select their genomes. Nat Rev Microbiol. 2005;3(8):643-655.

55. Dales S, Mosbach EH. Vaccinia as a model for membrane biogenesis. Virology. 1968;35(4):564-583.

56. Dancourt J, Barlowe C. Protein sorting receptors in the early secretory pathway. Annu Rev Biochem. 2010;79:777-802.

57. Dasgupta A, Wilson DW. ATP depletion blocks herpes simplex virus DNA packaging and capsid maturation. J Virol. 1999;73(3):2006-2015.

58. De Guzman RN, Wu ZR, Stalling CC, Pappalardo L, Borer PN, Summers MF. Structure of the HIV-1 nucleocapsid protein bound to the SL3 psi-RNA recognition element. Science. 1998;279(5349):384-388.

59. Deiss LP, Chou J, Frenkel N. Functional domains within the a sequence involved in the cleavage- packaging of herpes simplex virus DNA. J Virol. 1986;59(3):605-618.

60. Delmas O, Gardet A, Chwetzoff S, et al. Different ways to reach the top of a cell. Analysis of rotavirus assembly and targeting in human intestinal cells reveals an original raft-dependent, Golgi-independent apical targeting pathway. Virology. 2004;327(2):157-161.

61. Demirov DG, Freed EO. Retrovirus budding. Virus Res. 2004;106(2):87-102.

62. Deschambeault J, Lalonde JP, Cervantes-Acosta G, Lodge R, Cohen EA, Lemay G. Polarized human immunodeficiency virus budding in lymphocytes involves a tyrosine-based signal and favors cell-to-cell viral transmission. J Virol. 1999;73(6):5010-5017.

63. Dey D, Singh S, Khan S, et al. An extended motif in the SARS-CoV-2 spike modulates binding and release of host coatomer in retrograde trafficking. Commun Biol. 2022;5(1):115.

64. Di Paolo G, De Camilli P. Phosphoinositides in cell regulation and membrane dynamics. Nature. 2006;443(7112):651-657.

65. Dick RA, Mallery DL, Vogt VM, James LC. IP6 regulation of HIV capsid assembly, stability, and uncoating. Viruses. 2018;10(11):640.

66. Doedens JR, Giddings TH Jr, Kirkegaard K. Inhibition of endoplasmic reticulum-to-Golgi traffic by poliovirus protein 3A: genetic and ultrastructural analysis. J Virol. 1997;71(12):9054-9064.

67. Dou D, Revol R, Ostbye H, Wang H, Daniels R. Influenza A virus cell entry, replication, virion assembly and movement. Front Immunol. 2018;9:1581.

68. Draganova EB, Thorsen MK, Heldwein EE. Nuclear Egress. Curr Issues Mol Biol. 2021;41:125-170.

69. Duhaut SD, McCauley JW. Defective RNAs inhibit the assembly of influenza virus genome segments in a segment-specific manner. Virology. 1996;216(2):326-337.

70. Enami M, Sharma G, Benham C, Palese P. An influenza virus containing nine different RNA segments. Virology. 1991;185(1):291-298.

71. Evans DT, Serra-Moreno R, Singh RK, Guatelli JC. BST-2/tetherin: a new component of the innate immune response to enveloped viruses. Trends Microbiol. 2010;18(9):388-396.

72. Ewing SG, Byrd SA, Christensen JB, Tyler RE, Imperiale MJ. Ternary complex formation on the adenovirus packaging sequence by the IVa2 and L4 22-kilodalton proteins. J Virol. 2007;81(22):12450-12457.

73. Fabbretti E, Afrikanova I, Vascotto F, Burrone OR. Two non-structural rotavirus proteins, NSP2 and NSP5, form viroplasm-like structures in vivo. J Gen Virol. 1999;80(Pt 2):333-339.

74. Feng Z. Quasi-enveloped hepatitis virus assembly and release. Adv Virus Res. 2020;108:315-336.

75. Feng Z, Hirai-Yuki A, McKnight KL, Lemon SM. Naked viruses that aren't always naked: quasi-enveloped agents of acute hepatitis. Annu Rev Virol. 2014;1(1):539-560.

76. Forsell K, Xing L, Kozlovska T, Cheng RH, Garoff H. Membrane proteins organize a symmetrical virus. EMBO J. 2000;19(19):5081-5091.

77. Freed EO. HIV-1 gag proteins: diverse functions in the virus life cycle. Virology. 1998;251(1):1-15.

78. Fried H, Kutay U. Nucleocytoplasmic transport: taking an inventory. Cell Mol Life Sci. 2003;60(8):1659-1688.

79. Fujii K, Fujii Y, Noda T, et al. Importance of both the coding and the segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions. J Virol. 2005;79(6):3766-3774.

80. Garbitt-Hirst R, Kenney SP, Parent LJ. Genetic evidence for a connection between Rous sarcoma virus gag nuclear trafficking and genomic RNA packaging. J Virol. 2009;83(13):6790-6797.

81. Garoff H, Hewson R, Opstelten DJE. Virus maturation by budding. Microbiol Mol Biol Rev. 1998;62(4):1171-1190.

82. Garoff H, Sjoberg M, Cheng RH. Budding of alphaviruses. Virus Res. 2004;106(2):103-116.

83. Garrus JE, von Schwedler UK, Pornillos OW, et al. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell.

2001;107(1):55-65.

84. Ge P, Tsao J, Schein S, Green TJ, Luo M, Zhou ZH. Cryo-EM model of the bullet-shaped vesicular stomatitis virus. Science. 2010;327(5966):689-693.

85. Gelderblom HR, Ozel M, Pauli G. Morphogenesis and morphology of HIV. Structure-function relations. Arch Virol. 1989;106(1-2):1-13.

86. Gillespie LK, Hoenen A, Morgan G, Mackenzie JM. The endoplasmic reticulum provides the membrane platform for biogenesis of the flavivirus replication complex. J Virol. 2010;84(20):10438-10447.

87. Goepfert PA, Shaw K, Wang G, Bansal A, Edwards BH, Mulligan MJ. An endoplasmic reticulum retrieval signal partitions human foamy virus maturation to intracytoplasmic membranes. J Virol. 1999;73(9):7210-7217.

88. Gomez-Navarro N, Miller E. Protein sorting at the ER-Golgi interface. J Cell Biol. 2016;215(6):769-778.

89. Gong M, Kieff E. Intracellular trafficking of two major Epstein-Barr virus glycoproteins, gp350/220 and gp110. J Virol. 1990;64(4):1507-1516.

90. Gottlinger HG, Dorfman T, Sodroski JG, Haseltine WA. Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. Proc Natl Acad Sci U S A. 1991;88(8):3195-3199.

91. Grable M, Hearing P. cis and trans requirements for the selective packaging of adenovirus type 5 DNA. J Virol. 1992;66(2):723-731.

92. Granzow H, Klupp BG, Fuchs W, Veits J, Osterrieder N, Mettenleiter TC. Egress of alphaherpesviruses: comparative ultrastructural study. J Virol. 2001;75(8):3675-3684.

93. Griffiths G, Rottier P. Cell biology of viruses that assemble along the biosynthetic pathway. Semin Cell Biol. 1992;3(5):367-381.

94. Gudleski N, Flanagan JM, Ryan EP, Bewley MC, Parent LJ. Directionality of nucleocytoplasmic transport of the retroviral gag protein depends on sequential binding of karyopherins and viral RNA. Proc Natl Acad Sci U S A. 2010;107(20):9358-9363.

95. Hammarskjold ML, Winberg G. Encapsidation of adenovirus 16 DNA is directed by a small DNA sequence at the left end of the genome. Cell. 1980;20(3):787-795.

96. Hassan Z, Kumar ND, Reggiori F, Khan G. How viruses hijack and modify the secretory transport pathway. Cells. 2021;10(10):2535.

97. Hearing P, Samulski RJ, Wishart WL, Shenk T. Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. J Virol. 1987;61(8):2555-2558.

98. Hegde RS, Keenan RJ. The mechanisms of integral membrane protein biogenesis. Nat Rev Mol Cell Biol. 2022;23(2):107-124.

99. Hellen CUT, Wimmer E. Enterovirus structure and assembly. In: Robart HA, ed. Human Enterovirus Infections. American Society for Microbiology; 1995:155-174.

100. Heuser J. Deep-etch EM reveals that the early poxvirus envelope is a single membrane bilayer stabilized by a geodetic “honeycomb” surface coat. J Cell Biol. 2005;169(2):269-283.

101. Hogue BG, Machamer CE. Coronavirus structural proteins and virus assembly. In: Perlman S, Gallaher T, Snijder EJ, eds. Nidoviruses. ASM; 2007:179-200.

102. Hollinshead M, Vanderplasschen A, Smith GL, Vaux DJ. Vaccinia virus intracellular mature virions contain only one lipid membrane. J Virol. 1999;73(2):1503-1517.

103. Hong W. SNAREs and traffic. Biochim Biophys Acta. 2005;1744(3):493-517.

104. Hsieh SC, Liu IJ, King CC, Chang GJ, Wang WK. A strong endoplasmic reticulum retention signal in the stem-anchor region of envelope glycoprotein of dengue virus type 2 affects the production of virus-like particles. Virology. 2008;374(2):338-350.

105. Hulswit RJG, Paesen GC, Bowden TA, Shi X. Recent advances in bunyavirus glycoprotein research: precursor processing, receptor binding and structure. Viruses. 2021;13(2):353.

106. Husain M, Weisberg AS, Moss B. Existence of an operative pathway from the endoplasmic reticulum to the immature poxvirus membrane. Proc Natl Acad Sci U S A. 2006;103(51):19506-19511.

107. Irurzun A, Perez L, Carrasco L. Involvement of membrane traffic in the replication of poliovirus genomes: effects of brefeldin A. Virology. 1992;191(1):166-175.

108. Jackson MR, Nilsson T, Peterson PA. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J. 1990;9(10):3153-3162.

109. Jackson WT, Giddings TH Jr, Taylor MP, et al. Subversion of cellular autophagosomal machinery by RNA viruses. PLoS Biol. 2005;3(5):e156.

110. Jahn R, Sudhof TC. Membrane fusion and exocytosis. Annu Rev Biochem. 1999;68:863-911.

111. Jayakar HR, Jeetendra E, Whitt MA. Rhabdovirus assembly and budding. Virus Res. 2004;106(2):117-132.

112. Jennings BC, Kornfeld S, Doray B. A weak COPI binding motif in the cytoplasmic tail of SARS-CoV-2 spike glycoprotein is necessary for its

cleavage, glycosylation, and localization. FEBS Lett. 2021;595(13):1758-1767.

113. Jin J, Li F, Mothes W. Viral determinants of polarized assembly for the murine leukemia virus. J Virol. 2011;85(15):7672-7682.

114. Jose J, Snyder JE, Kuhn RJ. A structural and functional perspective of alphavirus replication and assembly. Future Microbiol. 2009;4(7):837-856.

115. Jouvenet N, Bieniasz PD, Simon SM. Imaging the biogenesis of individual HIV-1 virions in live cells. Nature. 2008;454(7201):236-240.

116. Jouvenet N, Neil SJ, Zhadina M, et al. Broad-spectrum inhibition of retroviral and filoviral particle release by tetherin. J Virol. 2009;83(4):1837-1844.

117. Jouvenet N, Simon SM, Bieniasz PD. Imaging the interaction of HIV-1 genomes and Gag during assembly of individual viral particles. Proc Natl Acad Sci U S A. 2009;106(45):19114-19119.

118. Jouvenet N, Zhadina M, Bieniasz PD, Simon SM. Dynamics of ESCRT protein recruitment during retroviral assembly. Nat Cell Biol. 2011;13(4):394-401.

119. Kabcenell AK, Poruchynsky MS, Bellamy AR, Greenberg HB, Atkinson PH. Two forms of VP7 are involved in assembly of SA11 rotavirus in endoplasmic reticulum. J Virol. 1988;62(8):2929-2941.

120. Kalderon D, Roberts BL, Richardson WD, Smith AE. A short amino acid sequence able to specify nuclear location. Cell. 1984;39(3 Pt 2):499-509.

121. Karayan L, Gay B, Gerfaux J, Boulanger PA. Oligomerization of recombinant penton base of adenovirus type 2 and its assembly with fiber in baculovirus-infected cells. Virology. 1994;202(2):782-795.

122. Kim DY, Firth AE, Atasheva S, Frolova EI, Frolov I. Conservation of a packaging signal and the viral genome RNA packaging mechanism in alphavirus evolution. J Virol. 2011;85(16):8022-8036.

123. Klein S, Cortese M, Winter SL, et al. SARS-CoV-2 structure and replication characterized by in situ cryo-electron tomography. Nat Commun. 2020;11(1):5885.

124. Klupp BG, Granzow H, Keil GM, Mettenleiter TC. The capsid-associated UL25 protein of the alphaherpesvirus pseudorabies virus is nonessential for cleavage and encapsidation of genomic DNA but is required for nuclear egress of capsids. J Virol. 2006;80(13):6235-6246.

125. Kohler A, Hurt E. Exporting RNA from the nucleus to the cytoplasm. Nat Rev Mol Cell Biol. 2007;8(10):761-773.

126. Kosinski J, Mosalaganti S, von Appen A, et al. Molecular architecture of the inner ring scaffold of the human nuclear pore complex. Science. 2016;352(6283):363-365.

127. Krijnse-Locker J, Schleich S, Rodriguez D, Goud B, Snijder EJ, Griffiths G. The role of a 21-kDa viral membrane protein in the assembly of vaccinia virus from the intermediate compartment. J Biol Chem. 1996;271(25):14950-14958.

128. Kuehn MJ, Herrmann JM, Schekman R. COPII-cargo interactions direct protein sorting into ER-derived transport vesicles. Nature. 1998;391(6663):187-190.

129. Lee JY, Cortese M, Haselmann U, et al. Spatiotemporal coupling of the hepatitis C virus replication cycle by creating a lipid droplet-proximal membranous replication compartment. Cell Rep. 2019;27(12):3602-3617 e5.

130. Lerner G, Weaver N, Anokhin B, Spearman P. Advances in HIV-1 assembly. Viruses. 2022;14(3):478.

131. Leroy H, Han M, Woottum M, et al. Virus-mediated cell-cell fusion. Int J Mol Sci. 2020;21(24):9644.

132. Liang Y, Huang T, Ly H, Parslow TG. Mutational analyses of packaging signals in influenza virus PA, PB1, and PB2 genomic RNA segments. J Virol. 2008;82(1):229-236.

133. Lim KP, Liu DX. The missing link in coronavirus assembly. Retention of the avian coronavirus infectious bronchitis virus envelope protein in the pre-Golgi compartments and physical interaction between the envelope and membrane proteins. J Biol Chem. 2001;276(20):17515-17523.

134. Lin DH, Stuwe T, Schilbach S, et al. Architecture of the symmetric core of the nuclear pore. Science. 2016;352(6283):aaf1015.

135. Lindemann D, Goepfert PA. The foamy virus envelope glycoproteins. Curr Top Microbiol Immunol. 2003;277:111-129.

136. Liu C, Eichelberger MC, Compans RW, Air GM. Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. J Virol. 1995;69(2):1099-1106.

137. Lodge R, Delamarre L, Lalonde JP, et al. Two distinct oncornaviruses harbor an intracytoplasmic tyrosine-based basolateral targeting signal in their viral envelope glycoprotein. J Virol. 1997;71(7):5696-5702.

138. Lontok E, Corse E, Machamer CE. Intracellular targeting signals contribute to localization of coronavirus spike proteins near the virus assembly site. J Virol. 2004;78(11):5913-5922.

139. Lopez T, Camacho M, Zayas M, et al. Silencing the morphogenesis of rotavirus. J Virol. 2005;79(1):184-192.

140. Lu K, Heng X, Summers MF. Structural determinants and mechanism of HIV-1 genome packaging. J Mol Biol. 2011;410(4):609-633.

141. Luytjes W, Krystal M, Enami M, Pavin JD, Palese P. Amplification, expression, and packaging of foreign gene by influenza virus. Cell. 1989;59(6):1107-1113.

142. Lv Y, Zhou S, Gao S, Deng H. Remodeling of host membranes during herpesvirus assembly and egress. Protein Cell. 2019;10(5):315-326.

143. Maeda J, Maeda A, Makino S. Release of coronavirus E protein in membrane vesicles from virus-infected cells and E protein-expressing cells. Virology. 1999;263(2):265-272.

144. Mancini EJ, Clarke M, Gowen BE, Rutten T, Fuller SD. Cryo-electron microscopy reveals the functional organization of an enveloped virus, Semliki Forest virus. Mol Cell. 2000;5(2):255-266.

145. Mangel WF, Baniecki ML, McGrath WJ. Specific interactions of the adenovirus proteinase with the viral DNA, an 11-amino-acid viral peptide, and the cellular protein actin. Cell Mol Life Sci. 2003;60(11):2347-2355.

146. Martin K, Helenius A. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. Cell. 1991;67(1):117-130.

147. Martin-Serrano J, Neil SJ. Host factors involved in retroviral budding and release. Nat Rev Microbiol. 2011;9(7):519-531.

148. Maruri-Avidal L, Domi A, Weisberg AS, Moss B. Participation of vaccinia virus l2 protein in the formation of crescent membranes and immature virions. J Virol. 2011;85(6):2504-2511.

149. Masters PS. Coronavirus genomic RNA packaging. Virology. 2019;537:198-207.

150. Mattei S, Schur FK, Briggs JA. Retrovirus maturation—an extraordinary structural transformation. Curr Opin Virol. 2016;18:27-35.

151. McBride MS, Panganiban AT. The human immunodeficiency virus type 1 encapsidation site is a multipartite RNA element composed of functional hairpin structures [published erratum appears in J Virol 1997 Jan;71(1):858]. J Virol. 1996;70(5):2963-2973.

152. McCown MF, Pekosz A. Distinct domains of the influenza a virus M2 protein cytoplasmic tail mediate binding to the M1 protein and facilitate infectious virus production. J Virol. 2006;80(16):8178-8189.

153. Mebatsion T, Konig M, Conzelmann KK. Budding of rabies virus particles in the absence of the spike glycoprotein. Cell. 1996;84(6):941-951.

154. Mebatsion T, Weiland F, Conzelmann KK. Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G. J Virol. 1999;73(1):242-250.

155. Melia CE, Peddie CJ, de Jong AWM, et al. Origins of enterovirus replication organelles established by whole-cell electron microscopy. MBio. 2019;10(3):e00951-19.

156. Mendes A, Kuhn RJ. Alphavirus nucleocapsid packaging and assembly. Viruses. 2018;10(3):138.

157. Mendonca L, Howe A, Gilchrist JB, et al. Correlative multi-scale cryo-imaging unveils SARS-CoV-2 assembly and egress. Nat Commun. 2021;12(1):4629.

158. Meng B, Lever AML. The interplay between ESCRT and viral factors in the enveloped virus life cycle. Viruses. 2021;13(2):324.

159. Mettenleiter TC. Herpesvirus assembly and egress. J Virol. 2002;76(4):1537-1547.

160. Mettenleiter TC, Klupp BG, Granzow H. Herpesvirus assembly: an update. Virus Res. 2009;143(2):222-234.

161. Meyer BE, Malim MH. The HIV-1 Rev trans-activator shuttles between the nucleus and the cytoplasm. Genes Dev. 1994;8(13):1538-1547.

162. Meyer JC, Bergmann CC, Bellamy AR. Interaction of rotavirus cores with the nonstructural glycoprotein NS28. Virology. 1989;171(1):98-107.

163. Milles S, Mercadante D, Aramburu IV, et al. Plasticity of an ultrafast interaction between nucleoporins and nuclear transport receptors. Cell. 2015;163(3):734-745.

164. Miyanari Y, Atsuzawa K, Usuda N, et al. The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol. 2007;9(9):1089-1097.

165. Moore MD, Fu W, Nikolaitchik O, Chen J, Ptak RG, Hu WS. Dimer initiation signal of human immunodeficiency virus type 1: its role in partner selection during RNA copackaging and its effects on recombination. J Virol. 2007;81(8):4002-4011.

166. Moore MD, Nikolaitchik OA, Chen J, Hammarskjold ML, Rekosh D, Hu WS. Probing the HIV-1 genomic RNA trafficking pathway and dimerization by genetic recombination and single virion analyses. PLoS Pathog. 2009;5(10):e1000627.

167. Morita E, Sandrin V, Chung HY, et al. Human ESCRT and ALIX proteins interact with proteins of the midbody and function in cytokinesis. EMBO J. 2007;26(19): 4215-4227.

168. Morita E, Sandrin V, McCullough J, Katsuyama A, Baci Hamilton I, Sundquist WI. ESCRT-III protein requirements for HIV-1 budding. Cell Host Microbe. 2011;9(3): 235-242.

169. Morita E, Sundquist WI. Retrovirus budding. Annu Rev Cell Dev Biol. 2004;20:395-425.

170. Mukhopadhyay S, Zhang W, Gabler S, et al. Mapping the structure and function of the E1 and E2 glycoproteins in alphaviruses. Structure.

2006;14(1):63-73.

171. Munro S, Pelham HR. A C-terminal signal prevents secretion of luminal ER proteins. Cell. 1987;48(5):899-907.

172. Mutsafi Y, Altan-Bonnet N. Enterovirus transmission by secretory autophagy. Viruses. 2018;10(3):139.

173. Naim HY, Roth MG. Basis for selective incorporation of glycoproteins into the influenza virus envelope. J Virol. 1993;67(8):4831-4841.

174. Nakielny S, Dreyfuss G. Nuclear export of proteins and RNAs. Curr Opin Cell Biol. 1997;9(3):420-429.

175. Narayanan K, Maeda A, Maeda J, Makino S. Characterization of the coronavirus m protein and nucleocapsid interaction in infected cells. J Virol. 2000;74(17):8127-8134.

176. Nayak DP, Balogun RA, Yamada H, Zhou ZH, Barman S. Influenza virus morphogenesis and budding. Virus Res. 2009;143(2):147-161.

177. Nayak DP, Hui EK, Barman S. Assembly and budding of influenza virus. Virus Res. 2004;106(2):147-165.

178. Neil SJ, Zang T, Bieniasz PD. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. Nature. 2008;451(7177):425-430.

179. Newcomb WW, Brown JC. Structure of the herpes simplex virus capsid: effects of extraction with guanidine hydrochloride and partial reconstitution of extracted capsids. J Virol. 1991;65(2):613-620.

180. Newcomb WW, Homa FL, Brown JC. Involvement of the portal at an early step in herpes simplex virus capsid assembly. J Virol. 2005;79(16):10540-10546.

181. Newcomb WW, Homa FL, Thomsen DR, et al. Assembly of the herpes simplex virus capsid: characterization of intermediates observed during cell-free capsid formation. J Mol Biol. 1996;263(3):432-446.

182. Newcomb WW, Juhas RM, Thomsen DR, et al. The UL6 gene product forms the portal for entry of DNA into the herpes simplex virus capsid. J Virol. 2001;75(22):10923-10932.

183. Newcomb WW, Trus BL, Cheng N, et al. Isolation of herpes simplex virus procapsids from cells infected with a protease-deficient mutant virus. J Virol. 2000;74(4):1663-1673.

184. Nicholls CMR, Sevvana M, Kuhn RJ. Structure-guided paradigm shifts in flavivirus assembly and maturation mechanisms. Adv Virus Res. 2020;108:33-83.

185. Noda T. Selective genome packaging mechanisms of influenza A viruses. Cold Spring Harb Perspect Med. 2021;11(7):a038497.

186. Noda T, Kawaoka Y. Structure of influenza virus ribonucleoprotein complexes and their packaging into virions. Rev Med Virol. 2010;20(6):380-391.

187. Novelli A, Boulanger PA. Deletion analysis of functional domains in baculovirus-expressed adenovirus type 2 fiber. Virology. 1991;185(1):365-376.

188. Nyathi Y, Wilkinson BM, Pool MR. Co-translational targeting and translocation of proteins to the endoplasmic reticulum. Biochim Biophys Acta. 2013;1833(11):2392-2402.

189. O'Brien JA, Taylor JA, Bellamy AR. Probing the structure of rotavirus NSP4: a short sequence at the extreme C terminus mediates binding to the inner capsid particle. J Virol. 2000;74(11):5388-5394.

190. Ono A, Ablan SD, Lockett SJ, Nagashima K, Freed EO. Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane. Proc Natl Acad Sci U S A. 2004;101(41):14889-14894.

191. Ono A, Orenstein JM, Freed EO. Role of the Gag matrix domain in targeting human immunodeficiency virus type 1 assembly. J Virol. 2000;74(6):2855-2866.

192. Orci L, Glick BS, Rothman JE. A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. Cell. 1986;46(2):171-184.

193. Ostapchuk P, Almond M, Hearing P. Characterization of empty adenovirus particles assembled in the absence of a functional adenovirus IVa2 protein. J Virol. 2011;85(11):5524-5531.

194. Ostapchuk P, Anderson ME, Chandrasekhar S, Hearing P. The L4 22-kilodalton protein plays a role in packaging of the adenovirus genome. J Virol. 2006;80(14):6973-6981.

195. Ostapchuk P, Hearing P. Control of adenovirus packaging. J Cell Biochem. 2005;96(1):25-35.

196. Ostapchuk P, Yang J, Auffarth E, Hearing P. Functional interaction of the adenovirus IVa2 protein with adenovirus type 5 packaging sequences. J Virol. 2005;79(5):2831-2838.

197. Paci G, Caria J, Lemke EA. Cargo transport through the nuclear pore complex at a glance. J Cell Sci. 2021;134(2):jcs247874.

198. Papa G, Borodavka A, Desselberger U. Viroplasms: assembly and functions of rotavirus replication factories. Viruses. 2021;13(7):1349.

199. Parent LJ, Bennett RP, Craven RC, et al. Positionally independent and exchangeable late budding functions of the Rous sarcoma virus and human immunodeficiency virus Gag proteins. J Virol. 1995;69(9):5455-5460.

200. Parent LJ, Gudleski N. Beyond plasma membrane targeting: role of the MA domain of Gag in retroviral genome encapsidation. J Mol Biol. 2011;410(4):553-564.

201. Parker SD, Hunter E. Activation of the Mason-Pfizer monkey virus protease within immature capsids in vitro. Proc Natl Acad Sci U S A. 2001;98(25):14631-14636.

202. Patton JT, Vasquez-Del Carpio R, Tortorici MA, Taraporewala ZF. Coupling of rotavirus genome replication and capsid assembly. Adv Virus Res. 2007;69:167-201.

203. Pereira LE, Clark J, Grznarova P, et al. Direct evidence for intracellular anterograde co-transport of M-PMV Gag and Env on microtubules. Virology. 2014;449:109-119.

204. Perez-Romero P, Tyler RE, Abend JR, Dus M, Imperiale MJ. Analysis of the interaction of the adenovirus L1 52/55-kilodalton and IVa2 proteins with the packaging sequence in vivo and in vitro. J Virol. 2005;79(4):2366-2374.

205. Pfister T, Pasamontes L, Troxler M, Egger D, Bienz K. Immunocytochemical localization of capsid-related particles in subcellular fractions of poliovirus-infected cells. Virology. 1992;188(2):676-684.

206. Pickl WF, Pimentel-Muinos FX, Seed B. Lipid rafts and pseudotyping. J Virol. 2001;75(15):7175-7183.

207. Pinto LH, Holsinger LJ, Lamb RA. Influenza virus M2 protein has ion channel activity. Cell. 1992;69(3):517-528.

208. Poruchynsky MS, Maass DR, Atkinson PH. Calcium depletion blocks the maturation of rotavirus by altering the oligomerization of virus-encoded proteins in the ER. J Cell Biol. 1991;114(4):651-656.

209. Posor Y, Jang W, Haucke V. Phosphoinositides as membrane organizers. Nat Rev Mol Cell Biol. 2022;23(12):797-816.

210. Raamsman MJ, Locker JK, de Hooge A, et al. Characterization of the coronavirus mouse hepatitis virus strain A59 small membrane protein E [In Process Citation]. J Virol. 2000;74(5):2333-2342.

211. Ramsey J, Mukhopadhyay S. Disentangling the frames, the state of research on the alphavirus 6K and TF proteins. Viruses. 2017;9(8):228.

212. Raveh B, Karp JM, Sparks S, et al. Slide-and-exchange mechanism for rapid and selective transport through the nuclear pore complex. Proc Natl Acad Sci U S A. 2016;113(18):E2489-E2497.

213. Reynolds AE, Wills EG, Roller RJ, Ryckman BJ, Baines JD. Ultrastructural localization of the herpes simplex virus type 1 UL31, UL34, and US3 proteins suggests specific roles in primary envelopment and egress of nucleocapsids. J Virol. 2002;76(17):8939-8952.

214. Riedel C, Hennrich AA, Conzelmann KK. Components and architecture of the rhabdovirus ribonucleoprotein complex. Viruses. 2020;12(9):959.

215. Risco C, Rodriguez JR, Lopez-Iglesias C, Carrascosa JL, Esteban M, Rodriguez D. Endoplasmic reticulum-Golgi intermediate compartment membranes and vimentin filaments participate in vaccinia virus assembly. J Virol. 2002;76(4):1839-1855.

216. Rixon FJ. Structure and assembly of herpesviruses. Semin Virol. 1993;4:135-144.

217. Rixon FJ, Addison C, McGregor A, et al. Multiple interactions control the intracellular localization of the herpes simplex virus type 1 capsid proteins. J Gen Virol. 1996;77(Pt 9):2251-2260.

218. Roberts A, Rose JK. Redesign and genetic dissection of the rhabdoviruses. Adv Virus Res. 1999;53:301-319.

219. Roberts KL, Smith GL. Vaccinia virus morphogenesis and dissemination. Trends Microbiol. 2008;16(10):472-479.

220. Robison CS, Whitt MA. The membrane-proximal stem region of vesicular stomatitis virus G protein confers efficient virus assembly [In Process Citation]. J Virol. 2000;74(5):2239-2246.

221. Rodriguez JF, Smith GL. IPTG-dependent vaccinia virus: identification of a virus protein enabling virion envelopment by Golgi membrane and egress. Nucleic Acids Res. 1990;18(18):5347-5351.

222. Rodriguez-Boulan E, Powell SK. Polarity of epithelial and neuronal cells. Annu Rev Cell Biol. 1992;8:395-427.

223. Roffman E, Albert JP, Goff JP, Frenkel N. Putative site for the acquisition of human herpesvirus 6 virion tegument. J Virol. 1990;64(12):6308-6313.

224. Rossman JS, Jing X, Leser GP, Lamb RA. Influenza virus M2 protein mediates ESCRT-independent membrane scission. Cell. 2010;142(6):902-913.

225. Rossman JS, Lamb RA. Influenza virus assembly and budding. Virology. 2011;411(2):229-236.

226. Rouiller I, Brookes SM, Hyatt AD, Windsor M, Wileman T. African swine fever virus is wrapped by the endoplasmic reticulum. J Virol. 1998;72(3):2373-2387.

227. Ruiz MC, Leon T, Diaz Y, Michelangeli F. Molecular biology of rotavirus entry and replication. ScientificWorldJournal. 2009;9:1476-1497.

228. Saad JS, Miller J, Tai J, Kim A, Ghanam RH, Summers MF. Structural basis for targeting HIV-1 Gag proteins to the plasma membrane for virus assembly. Proc Natl Acad Sci U S A. 2006;103(30):11364-11369.

229. Sadasivan J, Singh M, Sarma JD. Cytoplasmic tail of coronavirus spike protein has intracellular targeting signals. J Biosci.

2017;42(2):231-244.

230. Sakalian M, Hunter E. Molecular events in the assembly of retrovirus particles. Adv Exp Med Biol. 1998;440:329-339.

231. Sakurai A, Yasuda J, Takano H, Tanaka Y, Hatakeyama M, Shida H. Regulation of human T-cell leukemia virus type 1 (HTLV-1) budding by ubiquitin ligase Nedd4. Microbes Infect. 2004;6(2):150-156.

232. Salmons T, Kuhn A, Wylie F, et al. Vaccinia virus membrane proteins p8 and p16 are cotranslationally inserted into the rough endoplasmic reticulum and retained in the intermediate compartment. J Virol. 1997;71(10):7404-7420.

233. Salonen A, Ahola T, Kaariainen L. Viral RNA replication in association with cellular membranes. Curr Top Microbiol Immunol. 2005;285:139-173.

234. Sanchez-San Martin C, Liu CY, Kielian M. Dealing with low pH: entry and exit of alphaviruses and flaviviruses. Trends Microbiol. 2009;17(11):514-521.

235. Santiana M, Ghosh S, Ho BA, et al. Vesicle-cloaked virus clusters are optimal units for inter-organismal viral transmission. Cell Host Microbe. 2018;24(2):208-220 e8.

236. Sauter D, Schindler M, Specht A, et al. Tetherin-driven adaptation of Vpu and Nef function and the evolution of pandemic and nonpandemic HIV-1 strains. Cell Host Microbe. 2009;6(5):409-421.

237. Scheiffele P, Rietveld A, Wilk T, Simons K. Influenza viruses select ordered lipid domains during budding from the plasma membrane. J Biol Chem. 1999;274(4):2038-2044.

238. Schmid SI, Hearing P. Bipartite structure and functional independence of adenovirus type 5 packaging elements. J Virol. 1997;71(5):3375-3384.

239. Schmidt HB, Gorlich D. Transport selectivity of nuclear pores, phase separation, and membraneless organelles. Trends Biochem Sci. 2016;41(1):46-61.

240. Schuller AP, Wojtynek M, Mankus D, et al. The cellular environment shapes the nuclear pore complex architecture. Nature. 2021;598(7882):667-671.

241. Seitz S, Iancu C, Volz T, et al. A slow maturation process renders hepatitis B virus infectious. Cell Host Microbe. 2016;20(1):25-35.

242. Selzer L, Zlotnick A. Assembly and release of hepatitis B virus. Cold Spring Harb Perspect Med. 2015;5(12):a021394.

243. Sfakianos JN, LaCasse RA, Hunter E. The M-PMV cytoplasmic targeting-retention signal directs nascent Gag polypeptides to a pericentriolar region of the cell. Traffic. 2003;4(10):660-670.

244. Shimotohno K. HCV Assembly and Egress via modifications in host lipid metabolic systems. Cold Spring Harb Perspect Med. 2021;11(1):a036814.

245. Shirasaki T, Feng H, Duyvesteyn HME, et al. Nonlytic cellular release of hepatitis A virus requires dual capsid recruitment of the ESCRT-associated Bro1 domain proteins HD-PTP and ALIX. PLoS Pathog. 2022;18(8):e1010543.

246. Shomron O, Nevo-Yassaf I, Aviad T, et al. COPII collar defines the boundary between ER and ER exit site and does not coat cargo containers. J Cell Biol. 2021;220(6):e201907224.

247. Siu YL, Teoh KT, Lo J, et al. The M, E, and N structural proteins of the severe acute respiratory syndrome coronavirus are required for efficient assembly, trafficking, and release of virus-like particles. J Virol. 2008;82(22):11318-11330.

248. Skach WR. The expanding role of the ER translocon in membrane protein folding. J Cell Biol. 2007;179(7):1333-1335.

249. Smith GL, Law M. The exit of vaccinia virus from infected cells. Virus Res. 2004;106(2):189-197.

250. Smith GL, Vanderplasschen A, Law M. The formation and function of extracellular enveloped vaccinia virus. J Gen Virol. 2002;83(Pt 12):2915-2931.

251. Snyder JE, Azizgolshani O, Wu B, et al. Rescue of infectious particles from preassembled alphavirus nucleocapsid cores. J Virol. 2011;85(12):5773-5781.

252. Sodeik B, Krijnse-Locker J. Assembly of vaccinia virus revisited: de novo membrane synthesis or acquisition from the host? Trends Microbiol. 2002;10(1):15-24.

253. Stewart H, Bingham RJ, White SJ, et al. Identification of novel RNA secondary structures within the hepatitis C virus genome reveals a cooperative involvement in genome packaging. Sci Rep. 2016;6:22952.

254. Stirzaker SC, Whitfeld PL, Christie DL, Bellamy AR, Both GW. Processing of rotavirus glycoprotein VP7: implications for the retention of the protein in the endoplasmic reticulum. J Cell Biol. 1987;105(6 Pt 2):2897-2903.

255. Strack B, Calistri A, Craig S, Popova E, Gottlinger HG. AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding. Cell. 2003;114(6):689-699.

256. Strambio-De-Castillia C, Niepel M, Rout MP. The nuclear pore complex: bridging nuclear transport and gene regulation. Nat Rev Mol Cell Biol. 2010;11(7):490-501.

257. Strom AC, Weis K. Importin-beta-like nuclear transport receptors. Genome Biol. 2001;2(6):REVIEWS3008.

258. Sugrue RJ, Bahadur G, Zambon MC, Hall-Smith M, Douglas AR, Hay AJ. Specific structural alteration of the influenza haemagglutinin by amantadine. EMBO J. 1990;9(11):3469-3476.

259. Suhy DA, Giddings TH Jr, Kirkegaard K. Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: an autophagy-like origin for virus-induced vesicles. J Virol. 2000;74(19):8953-8965.

260. Swanstrom R, Wills JW. Synthesis, assembly and processing of viral proteins. In: Coffin JM, Hughes SH, Varmus HE, eds. Retroviruses. Cold Spring Harbor Laboratory Press; 1997:263-334.

261. Szajner P, Jaffe H, Weisberg AS, Moss B. A complex of seven vaccinia virus proteins conserved in all chordopoxviruses is required for the association of membranes and viroplasm to form immature virions. Virology. 2004;330(2):447-459.

262. Szajner P, Weisberg AS, Lebowitz J, Heuser J, Moss B. External scaffold of spherical immature poxvirus particles is made of protein trimers, forming a honeycomb lattice. J Cell Biol. 2005;170(6):971-981.

263. Takegami T, Kuhn RJ, Anderson CW, Wimmer E. Membrane-dependent uridylylation of the genome-linked protein VPg of poliovirus. Proc Natl Acad Sci U S A. 1983;80(24):7447-7451.

264. Taylor JA, O'Brien JA, Yeager M. The cytoplasmic tail of NSP4, the endoplasmic reticulum-localized non- structural glycoprotein of rotavirus, contains distinct virus binding and coiled coil domains. EMBO J. 1996;15(17):4469-4476.

265. Taylor MP, Kirkegaard K. Potential subversion of autophagosomal pathway by picornaviruses. Autophagy. 2008;4(3):286-289.

266. Thomas DC, Roth MG. The basolateral targeting signal in the cytoplasmic domain of glycoprotein G from vesicular stomatitis virus resembles a variety of intracellular targeting motifs related by primary sequence but having diverse targeting activities. J Biol Chem. 1994;269(22):15732-15739.

267. Tomishima MJ, Smith GA, Enquist LW. Sorting and transport of alpha herpesviruses in axons. Traffic. 2001;2(7):429-436.

268. Torrisi MR, Gentile M, Cardinali G, et al. Intracellular transport and maturation pathway of human herpesvirus 6. Virology. 1999;257(2):460-471.

269. Troxler M, Egger D, Pfister T, Bienz K. Intracellular localization of poliovirus RNA by in situ hybridization at the ultrastructural level using single-stranded riboprobes. Virology. 1992;191(2):687-697.

270. Ujike M, Taguchi F. Incorporation of spike and membrane glycoproteins into coronavirus virions. Viruses. 2015;7(4):1700-1725.

271. Van Damme N, Goff D, Katsura C, et al. The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. Cell Host Microbe. 2008;3(4):245-252.

272. Van den Berg B, Clemons WM Jr, Collinson I, et al. X-ray structure of a protein-conducting channel. Nature. 2004;427(6969):36-44.

273. van Genderen IL, Brandimarti R, Torrisi MR, Campadelli G, van Meer G. The phospholipid composition of extracellular herpes simplex virions differs from that of host cell nuclei. Virology. 1994;200(2):831-836.

274. Vennema H, Godeke GJ, Rossen JW, et al. Nucleocapsid-independent assembly of coronavirus-like particles by co-expression of viral envelope protein genes. EMBO J. 1996;15(8):2020-2028.

275. Vlach J, Lipov J, Rumlova M, et al. D-retrovirus morphogenetic switch driven by the targeting signal accessibility to Tctex-1 of dynein. Proc Natl Acad Sci U S A. 2008;105(30): 10565-10570.

276. Voorhees RM, Fernandez IS, Scheres SH, Hegde RS. Structure of the mammalian ribosome-Sec61 complex to 3.4 A resolution. Cell. 2014;157(7):1632-1643.

277. Voorhees RM, Hegde RS. Structure of the Sec61 channel opened by a signal sequence. Science. 2016;351(6268):88-91.

278. Waheed AA, Freed EO. The role of lipids in retrovirus replication. Viruses. 2010; 2(5):1146-1180.

279. Walter CT, Barr JN. Recent advances in the molecular and cellular biology of bunyaviruses. J Gen Virol. 2011;92(Pt 11):2467-2484.

280. Wang X, Ren J, Gao Q, et al. Hepatitis A virus and the origins of picornaviruses. Nature. 2015;517(7532):85-88.

281. Weigel AV, Chang CL, Shtengel G, et al. ER-to-Golgi protein delivery through an interwoven, tubular network extending from ER. Cell. 2021;184(9):2412-2429 e16.

282. Weisberg AS, Maruri-Avidal L, Bisht H, et al. Enigmatic origin of the poxvirus membrane from the endoplasmic reticulum shown by 3D imaging of vaccinia virus assembly mutants. Proc Natl Acad Sci U S A. 2017;114(51):E11001-E11009.

283. Weiss ER, Gottlinger H. The role of cellular factors in promoting HIV budding. J Mol Biol. 2011;410(4):525-533.

284. Welsch S, Miller S, Romero-Brey I, et al. Composition and three-dimensional architecture of the dengue virus replication and assembly sites. Cell Host Microbe. 2009;5(4):365-375.

285. Wodrich H, Guan T, Cingolani G, Von Seggern D, Nemerow G, Gerace L. Switch from capsid protein import to adenovirus assembly by cleavage of nuclear transport signals. EMBO J. 2003;22(23):6245-6255.

286. Wollert T, Hurley JH. Molecular mechanism of multivesicular body biogenesis by ESCRT complexes. Nature. 2010;464(7290):864-869.

287. Wright ER, Schooler JB, Ding HJ, et al. Electron cryotomography of immature HIV-1 virions reveals the structure of the CA and SP1 Gag shells. EMBO J. 2007;26(8):2218-2226.

288. Xie X, Zou J, Zhang X, et al. Dengue NS2A protein orchestrates virus assembly Cell Host Microbe. 2019;26(5):606-622 e8.

289. Yang P, Bansal A, Liu C, Air GM. Hemagglutinin specificity and neuraminidase coding capacity of neuraminidase-deficient influenza viruses. Virology. 1997;229(1):155-165.

290. Yasuda J, Hunter E, Nakao M, Shida H. Functional involvement of a novel Nedd4-like ubiquitin ligase on retrovirus budding. EMBO Rep. 2002;3(7):636-640.

291. Yu D, Weller SK. Herpes simplex virus type 1 cleavage and packaging proteins UL15 and UL28 are associated with B but not C capsids during packaging. J Virol. 1998;72(9): 7428-7439.

292. Yu IM, Hughson FM. Tethering factors as organizers of intracellular vesicular traffic. Annu Rev Cell Dev Biol. 2010;26:137-156.

293. Yu SF, Eastman SW, Linial ML. Foamy virus capsid assembly occurs at a pericentriolar region through a cytoplasmic targeting/retention signal in Gag. Traffic. 2006;7(8): 966-977.

294. Yuan B, Campbell S, Bacharach E, Rein A, Goff SP. Infectivity of Moloney murine leukemia virus defective in late assembly events is restored by late assembly domains of other retroviruses. J Virol. 2000;74(16):7250-7260.

295. Zeffman A, Hassard S, Varani G, Lever A. The major HIV-1 packaging signal is an extended bulged stem loop whose structure is altered on interaction with the gag polyprotein [In Process Citation]. J Mol Biol. 2000;297(4):877-893.

296. Zhang M, Ghosh S, Li M, Altan-Bonnet N, Shuai D. Vesicle-cloaked rotavirus clusters are environmentally persistent and resistant to free chlorine disinfection. Environ Sci Technol. 2022;56(12):8475-8484.

297. Zhang ZW, Li ZL, Yuan S. The role of secretory autophagy in Zika virus transfer through the placental barrier. Front Cell Infect Microbiol. 2016;6:206.

298. Zhao LJ, Padmanabhan R. Three basic regions in adenovirus DNA polymerase interact differentially depending on the protein context to function as bipartite nuclear localization signals. New Biol. 1991;3(11):1074-1088.

299. Zila V, Margiotta E, Turonova B, et al. Cone-shaped HIV-1 capsids are transported through intact nuclear pores. Cell. 2021;184(4):1032-1046 e18.

CHAPTER 7

Metabolism and Viral Infection

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Acknowledgments

Viruses have evolved as master manipulators of cellular processes. Many examples are documented of viral effects on signaling pathways, stress responses, transcription, translation, innate immunity, and metabolism.[60,72,108,135,144,159] All these virally mediated changes in cellular processes establish a cellular milieu that is supportive of infection. Although host cell metabolism is a key target for viral manipulation, it has been among the last of the major cellular processes to catch the attention of the virology field. However, studies of viral interactions with host metabolism are rapidly increasing in number and revealing fundamental knowledge of virus biology and virus-host interactions. These increases are being accelerated by advances in metabolomics technologies and the availability of metabolic assays, thus lowering the barrier to studying metabolism.

In this chapter, we will present and discuss basic metabolic pathways with a focus on virology, to provide a conceptual framework necessary for any virologist who wants to initiate or interpret metabolic studies of virus-host interactions. But we must be circumspect; while it is easy to believe in the infallibility of the metabolic pathways that have so long been laid out in biochemistry texts, we should only consider them for guidance, not dogma, since metabolism is dependent on so many aspects of the cell, the cell type, and the cellular environment.

Over the past 15-20 years our concepts of metabolism and its mechanisms have changed due to intense studies of cancer metabolism using both cell and animal models.[114,155,195] Indeed, findings in cancer metabolism have challenged many classical views of general metabolism. Cancer metabolism has become an important source of guidance in examining metabolism in virally infected cells. Examples of viral alterations of metabolism that mimic those seen during oncogenesis have been documented.[105,154] However, we must be prudent with this comparison; while many RNA and DNA viruses may alter some of the same metabolic pathways affected during oncogenesis, they may do so by very different mechanisms and for the production of different outcomes. Thus the study of virus-host interactions involving metabolism must mature as a field complementary to, but independent of, cancer metabolism.

The broad term metabolism considers both the breakdown (catabolism) of biomolecules and the building up (anabolism), or biosynthesis, of biomolecules. Catabolism transforms polysaccharides, nucleic acids, proteins, and lipids into their basic subunits, monosaccharides, nucleotides, amino acids, and fatty acids. Nutrients derived from catabolism, or imported from the extracellular environment, can be used in two ways for anabolic metabolism. First, the catabolic products can be oxidized to produce the energy needed for biosynthesis. Second, the basic subunits produced by catabolism can be used to build complex biomolecules. These catabolically produced building blocks supplement those that can be synthesized *de novo* by the cell. These aspects of metabolism, as well as the *de novo* synthetic pathways, provide the basic needs for viral productive infection. Hence, they are potential targets for manipulation during infection.

The metabolic program of a cell reflects the cell's function and its environment, including the controlled coordination between anabolism and catabolism as the cell transitions from quiescence to a proliferative state. It is easy to imagine how an infecting virus may need to activate metabolism in quiescent cells or fine-tune active metabolism in proliferative cells. Viruses need to establish conditions that produce sufficient energy and biosynthetic precursors to maintain the level of biosynthesis that is appropriate to support the success of the infection. These conditions will undoubtedly vary depending on whether the infection is productive, persistent, or latent.

Viruses have proven to be excellent experimental tools for examining the intricacies of the cell, often uncovering aspects of normal cell biology heretofore unknown, or little understood. In this same vein, metabolism studies in virally infected cells will likely provide deeper insight into the complex world of cellular metabolism and the many interconnections between metabolism and all other cellular processes.

In this chapter, we will highlight the basic cellular metabolic pathways of glycolysis, the tricarboxylic acid (TCA) cycle, glutaminolysis, and lipogenesis. We are not going to attempt to review the vast virology literature to exhaustively document viral effects on metabolism. Instead, we will discuss the metabolic pathways in terms of why viruses may want to affect them and at what key points in metabolism these effects would be targeted. Since the study of viral effects on metabolism is still relatively new, we will discuss and speculate on potential ways viruses may manipulate metabolism. We hope to provide thoughts and ideas for future experimentation, a guide to potential mechanisms and targets that should be considered by any virologist with an interest in metabolism. In this regard we will discuss approaches to be considered when planning metabolic studies. In this way we hope to aid the reader in considering metabolism in their viral studies. Since a great amount of our present understanding of metabolism in infected cells comes from studies using herpesviruses, we will highlight these studies and supplement them with findings from other viruses. We will be focusing primarily on mammalian metabolism but will highlight some important differences in viral infection in other host systems, we apologize to the nonanimal virologist for our animal virus centrism; we hope that you still find interesting aspects in the chapter.

We will proceed by considering the basic pathways. For those who are not well founded in metabolism, we will try to go through the pathways in an educational way tailored for virologists and virus studies. For those more familiar with metabolic pathways we find that it never hurts to reacquaint oneself with the pathways, as it often provides new insight. We will start with glycolysis.

Glycolysis

Glycolysis occurs in the cytoplasm and is the process of fermenting (no oxygen is required) a 6-carbon glucose molecule to two 3-carbon pyruvate molecules. Subsequently, the pyruvates can either be converted to lactate, acetate, alanine, or oxaloacetate (OAA) or, alternatively, enter the mitochondrial TCA cycle for further oxidation and ATP synthesis. In Figure 7.1 the glycolytic pathway is shown using the abbreviated names of the glycolytic intermediates between glucose and pyruvate; the full names of each intermediate are in the figure legend. Each reaction is catalyzed by a specific glycolytic enzyme. Some of the glycolytic reactions are reversible, while others are not. Blue bidirectional arrows indicate that the enzyme for that reaction can catalyze both the forward and reverse reactions. Red unidirectional arrows indicate the three glycolytic reactions that cannot be reversed; these enzymes can catalyze only the forward glycolytic reaction because they utilize ATP or involve the hydrolysis of a high-energy bond. The enzymes that catalyze the irreversible reactions are shown in Figure 7.1 because they provide points where glycolysis can be regulated and, therefore, are potential sites for direct or indirect viral effects. This will be discussed below.

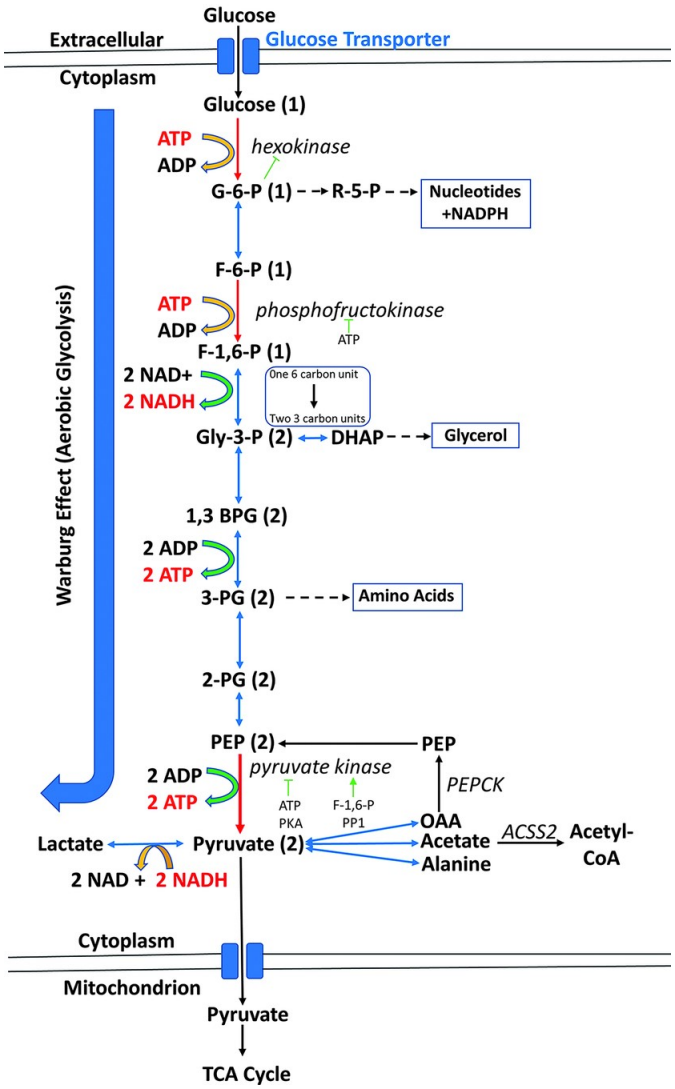

FIGURE 7.1 **The glycolytic pathway**. The steps converting glucose to pyruvate are indicated; each step is catalyzed by a specific glycolytic enzyme. *Blue bidirectional arrows* indicate that the enzyme can catalyze the forward and reverse directions to promote either glycolysis or gluconeogenesis. *Red unidirectional arrows* indicate where the enzyme can catalyze only the forward glycolytic reaction. The enzymes that catalyze the irreversible reactions (hexokinase, phosphofructokinase, and pyruvate kinase) are shown because they are targets for allosteric control or other types of control that modulate glycolytic activity. *Orange arrows* indicate the use of ATP or a reducing equivalent, NADH. *Green arrows* indicate the generation of ATP or NADH. Also shown are the glycolytic intermediates that can be drawn off for use in biosynthetic pathways, these are G-6-P, DHAP, 3-PG, and pyruvate. The *large blue arrow* indicates the Warburg effect or aerobic glycolysis, when the glucose-derived pyruvate is converted to lactate instead of entering the mitochondrion for further oxidation in the TCA cycle (Fig. 7.2). The gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) is shown and discussed in the text. The conversion of pyruvate-derived acetate to acetyl-CoA by ACSS2 is shown and discussed in the text. Abbreviations: G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P, fructose-1,6-bisphosphate; Gly-3-P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; R-5-P, ribose-5-phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; NAD+, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); NADPH, nicotinamide adenine dinucleotide phosphate (reduced); OAA, oxaloacetic acid; PKA, protein kinase A; PP1, protein phosphatase 1; PEPCK, phosphenolpyruvate carboxykinase; ACSS2, Acetyl-CoA synthetase short-chain family member 2.

In the process of gluconeogenesis, where glycolysis is reversed to make glucose from pyruvate, the three irreversible glycolytic reactions are catalyzed in the reverse direction by four gluconeogenesis-specific enzymes that are often expressed in a tissue-specific manner. It is not clear if, or why, a viral infection might induce gluconeogenesis. However, the gluconeogenic enzymes may play other roles in the cell which might be important for the viral infection. An example is phosphoenolpyruvate carboxykinase (PEPCK), which converts oxaloacetate (OAA) into phosphoenolpyruvate (PEP) (Fig. 7.1). PEPCK activity is greatly increased in human cytomegalovirus (HCMV)-infected fibroblasts,[18] suggesting that it may be used to convert cytoplasmic OAA to PEP (Fig. 7.1). However, PEPCK has another function; it activates the mechanistic target of rapamycin complex 1 (mTORC1) and this increases glycolysis.[98] HCMV infection dramatically increases mTORC1 activity and protects it from stress-induced inhibition[14,23-25,135]; thus PEPCK may be induced in HCMV infections to aid in mTORC1 activation. While this remains to be proven, the important consideration is that many of the metabolic enzymes may have other functions in the cell that may be important for viral infections. Another important point to consider is that the virally induced forms of metabolic enzymes may be tissue-specific isoforms that are not generally expressed in the infected tissue.[102] Their induction during infection may provide special functions or abilities that aid in the viral life cycle. We will return to the induction of tissue-specific isoforms when we discuss glucose transporters.

Returning to glycolysis, we can see in Figure 7.1 that it both uses and synthesizes ATP, as well as the reducing equivalent, NADH. NADH and its

oxidized counterpart NAD+ are co-enzymes used for either oxidation or reduction in many metabolic redox reactions, and they are needed for productive viral infections. Orange arrows indicate the use of ATP (ATP to ADP) or NADH (NADH to NAD+) in the ***forward glycolytic reactions, that is, glucose to pyruvate***. If the reaction can be reversed the opposite would occur (ADP to ATP or NAD+ to NADH). Green arrows indicate the generation of ATP (ADP to ATP) or NADH (NAD+ to NADH) in the forward reaction. Comparing the use and synthesis of ATP and NADH, we see that when one molecule of glucose is converted to two molecules of pyruvate there is a net gain of two ATPs and two NADHs. But there is so much more to this story.

Biosynthetic Pathways Using Glycolytic Intermediates

Not all of the glucose completes the pathway to pyruvate. Several glycolytic intermediates can be drawn off into important biosynthetic pathways. The products can be quite important during a productive viral infection:

1. *G-6-P (glucose-6-phosphate)* is converted to R-5-P (ribose-5-phosphate) in the pentose phosphate pathway that leads to nucleotide synthesis supporting cellular and viral RNA and DNA synthesis. Additionally, an important product of this pathway is the production of NADPH, another very important reducing equivalent used in many biosynthetic reactions, especially lipogenesis, which will be discussed in detail later in the chapter.

2. *Gly-3-P (glyceraldehyde-3-phosphate) and DHAP (dihydroxyacetone phosphate)* are the 3-carbon units that are produced by the breakdown of the 6-carbon F-1,6-P (fructose-1,6-phosphate). Gly-3-P and DHAP are easily interconverted. The important point is that DHAP can be drawn off for the synthesis of glycerol for lipid synthesis.

3. *3-PG (3-phosphoglycerate)* can be drawn off for the synthesis of nonessential amino acids (NEAAs), including l-serine. Besides its use as an amino acid, l-serine is a precursor for many other amino acids and metabolites, such as glycine, cysteine, tryptophan, phosphatidyl l-serine, sphingolipids, purines, porphyrins, glyoxalate, and glycine.

4. *Pyruvate* can be converted to alanine, lactate, acetate, and oxaloacetic acid (OAA) instead of entering the mitochondrial TCA cycle. As it turns out, all of these metabolites can be useful in productive viral infections. The conversion of pyruvate to acetate will be discussed further below since it is known to be used during HCMV infection.[167]

All of the products of glycolysis and its associated biosynthetic pathways (ATP, NADH, NADPH, nucleotides, glycerol, NEAAs, pyruvate, lactate, acetate, and OAA) are important for productive viral infections. Hence it is not difficult to see why glycolysis and its associated pathways are activated during infection by many viruses.[44,71,105,117,128] One of the first global analyses of metabolic flux, using ^{13}C-labeled glucose and mass spectrometry, examined glycolysis and the TCA cycle in cells infected with HCMV, a herpesvirus.[102,103] These data definitively showed that HCMV infection increased the uptake of glucose and increased glycolytic flux compared to mock-infected cells. In other words, labeled glucose was more rapidly converted into labeled glycolytic intermediates in HCMV-infected cells than in mock-infected cells. The data also suggested that the biosynthetic pathways using glycolytic intermediates are altered in infected cells: (1) Ribose-5-phosphate conversion into nucleotides was upregulated; (2) the intracellular pool of DHAP was increased, suggesting increased production of glycerol; (3) the intracellular pool of 3-PG was not dramatically increased, which left no clear prediction of whether this pathway to NEAAs was upregulated; (4) a significant amount of pyruvate was converted to alanine. However, an even greater amount of pyruvate was converted to lactate, a finding that we will discuss below. Additionally, a significant portion of the pyruvate entered the TCA cycle. In summary, these glycolytic flux data suggested that HCMV infection targets glycolysis and its associated biosynthetic reactions, and alters them to the advantage of the productive infection.

A Diversity in Metabolic Profiles

Several studies have found that related viruses may have widely divergent effects on the host metabolic profile, suggesting that virus-host metabolism interactions may be unique to each virus. Similar metabolic flux studies as described above for HCMV were done using a different herpesvirus, herpes simplex type 1 (HSV-1).[45,163] HSV-1 promotes the flow of carbons from glucose and glutamine to nucleotide synthesis,[45,163] while HCMV directs glucose towards lipid synthesis.[103,119,167,181,190] In another striking contrast, HCMV replication depends on argininosuccinate synthetase 1 (ASS1), but its enzymatic activity limits HSV-1 infection.[45] ASS1 pulls carbon and nitrogen atoms towards arginine synthesis and the urea cycle, limiting their flow to pyrimidine synthesis.[45] These observations suggest that nucleotide synthesis is a rate-limiting factor for HSV-1 infection but not HCMV. These studies in HSV-1 and HCMV were performed in lytic models and it is unknown how latent infection would influence metabolism. Further, Epstein-Barr virus (EBV) and Kaposi sarcoma herpesvirus (KSHV)—both gamma herpesviruses—alter host metabolism in ways that are also distinct from HCMV and HSV-1.[75] However, in comparing these studies we must consider the context in which these experiments were done. The metabolic effects of some herpesviruses, particularly the γ-herpesviruses, have mostly been studied in the context of latency. Latent γ-herpesviruses cause cancers, and oncogenesis requires host metabolic barriers that may limit proliferation to be overcome.[53,93] Some of the metabolic alterations are similar to metabolic profiles that happen in some cancers from nonviral etiology (eg, increase in aerobic glycolysis[11,31] and lipid synthesis,[32,171] and mitochondrial metabolism[127,170]). However, in one study of lytic replication of KSHV, infection one study of lytic replication of KSHV, infection promotes glycolysis similar to HSV-1 and HCMV infection, and enhances glutamine dependence and lipid metabolism like HCMV infection.[129] This highlighted work from the herpesvirus family demonstrates that the virus-host metabolism interaction is dependent on the virus, the cell type, and the type of infection (latent, persistent or productive).

It is important to remember that metabolic alterations that occur in virally infected cells reflect the natural history of both the virus and the host cell. Not only can related viruses have differing effects on metabolism, but cell types from the same host also have very different metabolic phenotypes. To highlight this point, consider how different tissues found in the human body have specialized metabolism. For example, while most cell types cannot make glucose from noncarbohydrate sources, hepatocytes can perform gluconeogenesis to produce glucose that can be distributed to other tissues.[153] In the brain, metabolism is partitioned by cell type reducing the competition for nutrients: neurons use glucose whereas other cells types in the brain consume lipids to generate energy.[66] Moreover, different cell types in the brain have intersecting metabolic networks. Astrocytes produce lactate that is released into the extracellular environment where neurons can import it to be fully metabolized via the TCA cycle after converting it to pyruvate.[86] This demonstrates that metabolism *in vivo* is a coordinated activity. During times of high energy demand, muscle cells will break down glycogen to lactate. The released lactate can be used to generate new glucose in the liver through gluconeogenesis, another example that tissues have coordinated metabolic activities. In the immune system different subtypes of T cells, B cells,

and macrophages have specialized metabolic profiles that are integral to their functions.[42,125,162]

It is important to remember that metabolism is also regulated by external signals including the nutrient environment that can be continuously changing in the body. As virologists, it is important to consider that not all cell types, especially in a given environment, will have metabolic phenotypes that are described in textbooks. Thus, continued studies of viruses, even related viruses, in relevant cell types are needed to understand how each virus uniquely interacts with host metabolism.

Controls of Glycolysis that may be Exploited by Viral Infections

Glycolysis, indeed, all the metabolic pathways in the cell, can be controlled at many levels.

Metabolic enzymes can be controlled at the transcriptional level by cell signaling pathways that may activate or inactivate transcription factors. Many of these signaling pathways are affected by growth factors. Viruses routinely alter aspects of cell signaling. Thus the effects that any specific virus has on growth factors and signaling must be considered for their potential effects on the metabolic transcriptome. These results can be correlated to metabolism and specific metabolic activation mechanisms predicted.

The ability of growth factors and their receptors to induce new transcription and translation led to the hypothesis that demands of cell growth were met through the replacement of nutrients as they are consumed during macromolecular synthesis, a demand-based system of nutrient uptake. However, this mechanism has been challenged.[156] There is increasing evidence from the cancer metabolism field to suggest a “supply side” mechanism where nutrient uptake is a “push mechanism.” In other words, there is direct stimulation of nutrient uptake in which growth factor signaling increases the uptake of glucose, lipids, and amino acids to levels that *exceed* a cell’s needs. This creates a pro-growth nutrient environment, which, together with signaling, can then push cells to grow. Under these conditions, adaptive changes in metabolism lead to the accumulation of anabolic precursors for the synthesis of protein, lipids, and nucleic acids. For some oncogenic viruses, metabolic alterations due to virus infection can help push cell growth and transformation. The metabolic changes that favor cell growth and oncogenesis may also stimulate virus replication as is the case for hepatitis C virus (HCV) or, alternatively, it may be independent of virus replication as is the case for EBV and KSHV, where the latent infection leads to changes in metabolism and oncogenesis.[118] A supply-side stiumlation of cell growt supply-side stimulation of cell growth could be an ideal mechanism to be exploited during viral infection to meet or exceed the energetic and biosynthetic needs of the infected cell. This may be particularly true for a virus with a very rapid productive lytic cycle. As an example of how a virus can attain nutrient excess to create a “pro-growth” state, let’s return to glycolysis and consider glucose uptake.

To initiate glycolysis, glucose must be brought into the cell. Figure 7.1 shows that this is accomplished by glucose transporters (GLUTs) located in the plasma membrane. GLUTs carry hexose sugars across the membrane without requiring energy. In eukaryotes GLUTs constitute a family of at least 13 members in three structural classes: GLUT1–4 (class I); GLUT5, 7, 9, and 11 (class II); and GLUT6, 8, 10, 12, and (H+)-myo-inositol cotransporter (class III).[70,160] Many of these are tissue specific or function best under specific conditions. Class I GLUTs are the best characterized, and for this discussion, we will consider two of them, the ubiquitously distributed GLUT1, which is abundant in fibroblasts, and GLUT4, which is very efficient and is the major GLUT in adipose tissue, skeletal muscle, and cardiac muscle.[62,76,100] During HCMV infection in fibroblasts, GLUT1 is quickly decreased to nearly undetectable levels and is replaced by the more efficient GLUT4, which is normally not detectable in fibroblasts.[189] This is mediated by the repression of GLUT1 gene transcription and activation of GLUT4 gene transcription. Thus the virus induces a more efficient transporter, but one that is not usually present in this cell type. The use of tissue-specific isoforms to support viral infections is a recurring theme as discussed above with respect to glycolysis. The evidence suggests that HCMV induces a tissue-specific isoform, GLUT4, that provides greater glucose uptake for the infected cell. It is an example of how supply-side stimulation can be accomplished, where the viral infection takes action to ensure increased nutrient uptake, making certain that levels meet or exceed the infected cell’s needs.

Protein modification is another way to affect the activity of metabolic enzymes. Activation of signaling pathways, or cell stress responses, induce kinases, phosphatases, and other protein modification enzymes. These enzymes modify metabolic enzymes to change their activity. A good example from glycolysis is pyruvate kinase (Fig. 7.1), which can be inactivated when phosphorylated by protein kinase A (PKA), and activated when dephosphorylated by phosphoprotein phosphatase 1 (PP1). Such enzymes can be influenced by many cellular signals and growth conditions affected by viral infection. The manipulation of such protein modification mechanisms during a viral infection provides another potential means for viral intervention in metabolic control.

Allosteric Regulation

Allosteric regulation is a very important concept in enzymology that is not always considered in virological studies, but we should consider why viruses might manipulate allosteric regulation in metabolism.

Many enzymes are regulated by the binding of an effector molecule at a regulatory site on the enzyme. The result of this binding is a conformational change in the enzyme that alters the enzyme’s activity. Effector molecules can activate or inhibit the enzyme’s activity. Allosteric regulation is a major means of controlling metabolic pathways; it also affects enzymes in nonmetabolic reactions, for example in cell signaling.[109,139]

Effector molecules are usually small molecules like ATP or a pathway-associated metabolite. Consider a metabolite in a metabolic pathway that is accumulating because it is not being readily used in the next step of the pathway. In order to slow this accumulation, the metabolite functions as an allosteric inhibitor of the enzyme that produces it. When the metabolite reaches a specific concentration, it can effectively bind the enzyme and change the enzyme’s conformation, inhibiting its enzymatic activity. This will slow the production of the metabolite and relieve its accumulation. Once the concentration of the metabolite decreases to a more homeostatic level, the allosteric inhibition is relieved and enzymatic activity returns. This is an example of feedback inhibition: the product of the reaction feeds back and controls the enzyme producing it. There are also examples of feedforward controls; examples of both feedback and feedforward controls will be discussed below.

It is important to keep in mind that allosteric controls can greatly activate or inhibit an enzyme without changing the amount of the enzyme in the cell. Thus, higher activity does not *necessarily* mean increased levels of the enzyme, and decreased activity does not *necessarily* mean lower levels. In most virology studies, it is routine to determine transcript and protein levels for a specific enzyme and correlate this with activity. In many cases this leap of faith is acceptable, but it may not be if the enzyme is allosterically controlled. For example, if one found that the level of an allosterically controlled enzyme decreased during a viral infection, one *could not* conclude that there would be less enzyme activity. Under the right allosteric conditions, the lower amount of enzyme may be capable of a great deal of enzymatic activity. Conversely, a high amount of

enzyme may have little activity under the influence of an allosteric inhibitor. At some point in the study of metabolic enzymes, the true enzymatic activity should be determined.

Allosteric Regulation of Glycolysis

The concept of allosteric regulation can be well demonstrated by its role in the regulation of glycolysis. As we discussed earlier, there are three irreversible reactions in the glycolytic pathway; these are the points of allosteric control. Figure 7.1 shows the three enzymes involved in these reactions: hexokinase, phosphofructokinase, and pyruvate kinase. Let's look at the allosteric control of each.

1. *Hexokinase* catalyzes the phosphorylation of glucose forming glycose-6-phosphate (G-6-P). It is controlled by feedback inhibition by the product of the reaction, G-6-P. If G-6-P levels increase because it is not being rapidly utilized in glycolysis or the pentose phosphate pathway, it will feedback and inhibit hexokinase, thereby allosterically inhibiting its production.

2. *Phosphofructokinase* (PFK) catalyzes the conversion of fructose-6-phosphate (F-6-P) to fructose-1,6-bisphosphate (F-1,6-P); it is considered the most important regulatory step in glycolysis. While the enzyme utilizes ATP it is also allosterically inhibited by ATP. Recall that ATP is a valuable product of glycolysis; thus it would be wasteful to overproduce ATP if it was not being used by the cell. What better way to avoid overproduction than by having the levels of ATP be sensed, allosterically, by PFK, slowing its activity and inhibiting glycolysis? Conversely, when ATP utilization is high, ADP, AMP, and inorganic phosphate (Pi) accumulate signaling the need for more ATP. Thus increased ADP and AMP counteracts the inhibition of phosphofructokinase by ATP, resulting in increased glycolysis. More broadly, the enzyme is being controlled by the cell's energetic environment. It senses the cellular ATP/ADP and ATP/AMP ratios; when the ratios are high, PFK activity is reduced, and when it is low, PFK activity is increased.

3. PFK is also allosterically inhibited by phosphoenolpyruvate (PEP), one of the last metabolites in the glycolytic pathway, just before pyruvate (Fig. 7.1). If PEP is accumulating at the end of the pathway it can feedback to control PFK at the beginning of glycolysis and turn down the entire pathway. Thus, PFK controls glycolysis allosterically by sensing not only the energic status of the entire cell but also the abundance of glycolytic metabolites.

4. *Pyruvate kinase* catalyzes the conversion of PEP to pyruvate with the production of ATP. It is allosterically inhibited by ATP, another example of an enzyme sensing the energetic environment of the cell. It is allosterically activated by F-1,6-P, an early metabolite in glycolysis. If F-1,6-P accumulates, it allosterically activates PK at the end of glycolysis, thus increasing the activity of the entire glycolytic pathway. This exemplifies a feed-forward allosteric mechanism.

The important point of this discussion, besides explaining allosteric controls, is that allosteric effects on enzymes have been little considered as a means of viral control. Yet a viral mechanism that alters allosteric controls may provide a great advantage for a viral infection. Viruses may have evolved means to block unwanted allosteric controls or enhance advantageous ones. Many viruses alter the compartmentalization of the infected cell, potentially protecting specific enzymes from allosteric inhibition or exposing them to advantageous allosteric activation.

Aerobic glycolysis, the Warburg effect

It is important to consider that the glucose flux analysis in HCMV-infected cells discussed above[102,103,163,172] showed that a significant proportion of the glucose-derived pyruvate was converted to lactic acid. Nearly a century ago, Otto Warburg observed that rapidly proliferating tumor cells consumed much more glucose than did most nontransformed tissues,[172] a condition now known as the Warburg effect or aerobic glycolysis (Fig. 7.1, large blue arrow). The conversion of glucose to lactate via pyruvate requires no oxygen; it is a fermentation reaction, and fermentation usually occurs under anaerobic conditions. The term aerobic glycolysis implies that the fermentation to lactate is occurring in the presence of abundant oxygen, specifically, at oxygen levels sufficient to support oxidative phosphorylation in the mitochondrion. If, instead of being converted to lactate, the pyruvate had entered the mitochondrion to enter the TCA cycle and oxidative phosphorylation, than much more ATP would have been produced from the original glucose molecule. This was the confusing aspect of the Warburg effect/aerobic glycolysis: why was the glucose not used to its full potential for ATP production in oxygenated, proliferating cells with an intact mitrochondrial functions? This question remains an important topic of study in cancer biology, normal cell biology, and now virology.

The other problem presented by aerobic glycolysis is the production of lactate, which must be secreted in order to maintain intracellular pH. Hence it was considered a waste product, and, again, a waste of the energy potential glucose could have supplied. It was thus confounding when it was discovered that aerobic glycolysis is actually a hallmark of proliferative metabolism in metazoan cells, not just a phenomenon of cancer cell proliferation.[84] This led many to consider that mitochondrial metabolism, the TCA cycle, and oxidative phosphorylation might not be as important for cell proliferation as previously supposed. This question remains, although more recent studies have shown that proliferative cells and tumor cells do increase flux through the TCA cycle, generating metabolites essential for biosynthesis.[36,131] Further, it was found that lactate can be taken up by many cell types, converted back to pyruvate, and used to fuel the TCA cycle in tumor cells as well as normal cells.[37,64] Given these findings, one can speculate that some viral infections may take advantage of the same processes. Excessive glycolysis leads to lactate production; the lactate is secreted to maintain intracellular pH but acidifies the infected cell microenvironment. This may aid in immune evasion and prepare surrounding cells for infection through uptake and utilization of lactate.

While the importance of both aerobic glycolysis and the TCA cycle could be established for proliferative cells, the question that arises is what is the division of labor between aerobic glycolysis and the TCA cycle. One possibility is that rapid aerobic glycolysis may be able to produce most of the ATP needed for rapid proliferation or viral infection,[99] leaving the major function of the TCA cycle to be the production of biosynthetic intermediated (discussed below) and not a major source of ATP. To make this mechanism successful, some ATP-expensive cellular processes may have to be curtailed. Many tumor cells dedifferentiate, thereby curtailing ATP-expensive processes, relieving the demands for ATP produced by aerobic glycolysis. In this regard, many viruses curtail cellular processes; for example, host shutoff mechanisms used by many viruses curtail the energy needs for host mRNA translation.[144] Additionally, some cells seem to survive and heal while dependent on aerobic glycolysis. After brain injury astrocytes inhibit the TCA cycle and initiate an aerobic glycolysis-like response as a restorative measure.[141] This is believed to be a protective mechanism to mitigate the oxidative damage that may result from reactive oxygen species (ROS) produced during mitochondrial oxidative phosphorylation. Perhaps viruses use a similar strategy; by relying on aerobic glycolysis rather than oxidative phosphorylation for ATP they limit the potential harmful effects of high levels of ROS on virus replication. This is a reasonable assumption since many viral infections increase aerobic glycolysis.[105,128,179,188] Many viruses, especially rapidly replicating ones, may rely completely, or in large part, on activated

aerobic glycolysis for their ATP needs under conditions of adequate glucose or glycogen availability. Thus leaving the TCA cycle to produce biosynthetic intermediates, possible utilizing glutamine instead of glucose (discussed below). Thus in virally infected cells showing increased aerobic glycolysis, the status of, or function of, the TCA cycle and oxidative phosphorylation should be determined. This will provide a more complete picture of how cellular metabolism has been altered to establish the energetic and biosynthetic milieu needed for the specific viral infection.

Finally, we are left with an intriguing question: Is there any virus that can rely completely on aerobic glycolysis to support its infectious cycle in the complete absence of the TCA cycle and oxidative phosphorylation? Recall that all of the products of glycolysis and its associated biosynthetic pathways (ATP, NADH, NADPH, nucleotides, glycerol, NEAAs, pyruvate, acetate, and OAA) are important for energy production and many aspects of biosynthesis. Is aerobic glycolysis, combined with other non-TCA/oxidative phosphorylation metabolic pathways, sufficient? It will be interesting to find out.

The Tricarboxylic Acid cycle

The TCA cycle (Fig. 7.2), also known as the citric acid cycle or the Krebs cycle, is a cyclic set of biochemical reactions that occur in the mitochondria. When glycolysis-derived pyruvate enters the mitochondrion, through the mitochondrial pyruvate carrier (MPC), it is converted to acetyl-coenzyme A (acetyl-CoA) by the pyruvate dehydrogenase multienzyme complex (PDH). At that point, the TCA cycle initiates when the two carbons of acetate from acetyl-CoA are combined with the four carbons of oxaloacetate (OAA) to form the six-carbon citrate molecule (Fig. 7.2).

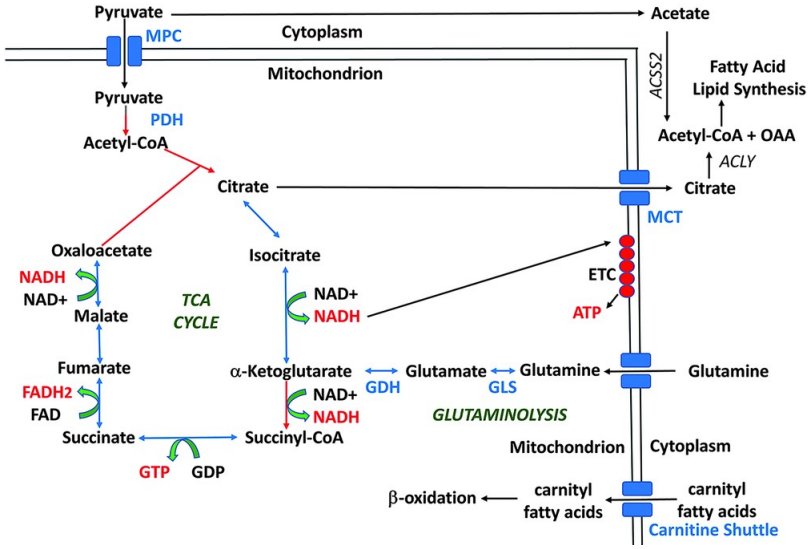

FIGURE 7.2 **The tricarboxylic acid cycle (TCA cycle)**. Glycolysis-derived pyruvate enters the mitochondrion through the mitochondrial pyruvate carrier (MPC) where it is converted to acetyl-coenzyme A (acetyl-CoA) by pyruvate dehydrogenase (PDH). The TCA cycle follows; all of the intermediates are shown; each reaction is catalyzed by a specific enzyme; irreversible reactions are shown by *red unidirectional arrows*, and reversible reaction are indicated by *blue bidirectional arrows*. The generation of reducing equivalents (NADH and FADH2) or GTP is shown by *green arrows*. All of the reducing equivalents can be used in the electron transport chain (ETC) for ATP production. Also shown is the transport of citrate from the mitochondrion to the cytoplasm by the mitochondrial citrate shuttle (MCS). In the cytoplasm citrate is converted into acetyl-CoA and oxaloacetate (OAA) by ATP-citrate lyase (ACLY). Also shown is the formation of acetyl-CoA from pyruvate-derived acetate by acetyl-CoA synthetase short-chain family member 2 (ACSS2). The steps of glutaminolysis indicate where glutamine is brought into the mitochondrion and converted to α-ketoglutarate by glutamine synthetase (GLS) and glutamate dehydrogenase (GDH).

Acetyl-CoA and Citrate

We should give some special consideration to acetyl-CoA and citrate because they define a critical branchpoint in the TCA cycle. Acetyl-CoA is the essential acetyl group donor in posttranslational acetylation reactions of proteins and other biomolecules; in addition, it is the source of acetyl groups for lipid synthesis. Thus acetyl-CoA is used in many parts of the cell. The problem is that mitochondrial acetyl-CoA cannot be exported from the mitochondria; however, citrate can be exported via the mitochondrial citrate shuttle (MCS, Fig. 7.2). There is an advantage and a disadvantage to this transport of citrate. The disadvantage is that a key metabolite for the TCA cycle is being removed and the forward progress of the TCA cycle is diminished; below we will discuss how this can be counteracted through the metabolism of glutamine. The advantage of the transport is that in the cytoplasm the citrate can be converted back to acetyl-CoA and OAA by ATP-citrate lyase (ACLY), providing cytoplasmic acetyl-CoA (Fig. 7.2).

Many studies have suggested that ACLY is the primary enzyme involved in making cytosolic acetyl-CoA.[7,56] However, it is important to note that there is another enzyme, acetyl-CoA synthetase short-chain family member 2 (ACSS2), that can make acetyl-CoA using free acetate (Figs. 7.1 and 7.2). Besides its important role in forming cytoplasmic acetyl-CoA, ACSS2 can also be transported to the nucleus where its acetyl-CoA synthetic activity can exert control on histone acetylation and, consequently, gene expression.[96] Clearly, control of this enzyme may be very important during a viral infection. In fact, it has been shown to be very important for HCMV infection.[178] ACLY and ACSS2 are both activated to produce acetyl-CoA for lipogenesis during HCMV infection. Interestingly, ACLY could be knocked out with no effect on lipogenesis or HCMV growth. Under these conditions, ACSS2 compensated for the loss of ACLY. Thus the viral infection can rely heavily on free acetate for acetyl-CoA synthesis. Free acetate has an important role in maintaining energy homeostasis in mammals, and it is known to be an essential carbon source for lipogenesis in cancer cells under stress conditions.[91,132] The sources of free acetate are many: it can be derived exogenously from foods or produced by the gut flora, it can be produced from ethanol metabolism, and it can be derived through the deacetylation of acetylated proteins

and acetylcholine, and through fatty acid β-oxidation. The latter two mechanisms could be exploited by viral infections for acetate production. Importantly, it had long been thought that there was no cellular metabolic mechanism for the production of acetate directly from glucose. However, in the HCMV study,[167] it was shown that glucose-derived pyruvate could be converted to free acetate and this mechanism was used in the infected cells. There are both a nonenzymatic[167] and an enzymatic means of performing this conversion.[83] Clearly, viruses that need an abundance of acetyl-CoA may be able to induce either of these mechanisms to allow ACSS2 to produce acetyl-CoA from acetate in the cytoplasm or the nucleus.

Besides a nuclear localization of ACSS2, it is important to note that PDH, the enzyme that produces mitochondrial acetyl-CoA, can also be found in the nucleus where it is also believed to provide acetyl-CoA for histone acetylation.[147] Although effects of viral infection on nuclear PDH or nuclear ACSS2 have not been examined, it is certainly intriguing to consider that a viral infection might manipulate the nuclear production of acetyl-CoA by either of these enzymes.

Reducing equivalents and oxidative phosphorylation. Let's return to the mitochondrion and think about the citrate that remains in the TCA cycle. The next step is its conversion to its isomer, isocitrate. This reaction is followed by two oxidative decarboxylation steps. First, the six-carbon isocitrate is converted into the five-carbon α-ketoglutarate and, second, to the four-carbon succinate unit in succinyl-CoA. The results of these reactions are the release of two molecules of CO_2 and the generation of two molecules of NADH.

The production of NADH marks the beginning of an important function of the TCA cycle, supplying reducing equivalents, redox factors, for the production of ATP by the electron transport chain (ETC) (Fig. 7.2). The ETC is a series of reactions that occur in the mitochondrial inner membrane. They are collectively known as oxidative phosphorylation, whereby the transport of electrons through the ETC generates a proton gradient across the inner mitochondrial membrane. The potential energy stored in the mitochondrial proton gradient is harnessed by ATP synthase to phosphorylate ADP to make ATP using oxygen.

All of the reducing equivalents made in the TCA cycle (NADH and FADH2, Fig. 7.2) can be utilized in oxidative phosphorylation in the ETC for ATP synthesis. The production of reducing equivalents in the TCA cycle continues in the reactions that follow from succinyl-CoA (Fig. 7.2). Succinyl-CoA is converted to succinate—producing CoA and GTP. The GTP can be converted to ATP without the loss of energy. The succinate is converted to fumarate with the production of flavin adenine dinucleotide (FADH2), another redox cofactor used by the ETC. In the final steps, fumarate is converted to malate and malate to OAA with the production of more NADH for oxidative phosphorylation. If we consider that all of the possible NADH and FADH2 molecules are produced in the TCA cycle from the two glycolysis-derived pyruvate molecules, then oxidative phosphorylation will generate 26 ATP molecules just from the reducing equivalents. This is why we made such a big deal about the apparent inefficiency of aerobic glycolysis; all of this ATP from oxidative phosphorylation would be lost. Again we must consider our above discussion, that high levels of ROS produced by oxidative phosphorylation may be too deleterious for some cellular and viral conditions. As mentioned above, the status and the importance of the TCA cycle and oxidative phosphorylation must be considered and determined on a virus-specific basis for it may vary greatly between different virus families and even between different viruses within a family.

Glutaminolysis

In the discussion of aerobic glycolysis, we noted that a consequence of converting glucose-derived pyruvate to lactic acid is that it lowers the amount of pyruvate that enters the mitochondrion, thus depriving the TCA cycle of glucose-derived carbon for the production of ATP. Similarly, the removal of citrate from the mitochondrion deprives the TCA cycle of an essential metabolite and again removes glucose-derived carbon from the cycle. Additionally, other TCA cycle metabolites can also be removed from the mitochondrion for a variety of purposes.[90] Each removal causes a loss of TCA cycle activity and reduced ATP production. However, there is a way to compensate by using exogenous glutamine, the most abundant amino acid in the body, and the second greatest source of carbon. As shown in Figure 7.2, glutamine can enter the mitochondrion where it is converted to glutamate by glutaminase (GLS) and glutamate to α-ketoglutarate by glutamate dehydrogenase (GDH). The resulting α-ketoglutarate then enters the TCA cycle. Thus glutamine fills the TCA cycle with glutamine-derived carbon to replace the lost glucose-derived carbon. The filling of a pathway in this way is called anaplerosis.

A number of viral infections are known to anaplerotically use glutamine to maintain the TCA cycle,[12,18,39,77,128,129,137] compensating for pyruvate being used in aerobic glycolysis and citrate being siphoning off for lipogenesis. For example, human cytomegalovirus is very dependent on glutaminolysis due to its upregulation of lipogenesis.[18]

The obvious use of glutaminolysis-derived α-ketoglutarate is for it to be converted to succinyl-CoA to continue the TCA cycle, thus supplying downstream TCA cycle intermediates and reducing equivalents for ATP synthesis. However, recall that the TCA cycle reactions that convert citrate to α-ketoglutarate (Figs. 7.2 and 7.3), catalyzed by aconitase 2 and isocitrate dehydrogenase 2, are reversible. Thus in the reverse reactions, α-ketoglutarate derived from glutamine could be converted back to citrate to support cytoplasmic acetyl-CoA production and lipogenesis. Importantly, this conversion of α-ketoglutarate to citrate can also be accomplished in the cytoplasm using cytoplasmic isoforms of the enzymes, aconitase 1 and isocitrate dehydrogenase 1 (Fig. 7.3). It is important to consider that this use of α-ketoglutarate, in either the mitochondrion or the cytoplasm, would be an ideal point for manipulation by a viral infection that requires increased lipogenesis, especially under conditions where aerobic glycolysis is depriving the TCA cycle of glucose-derived pyruvate.

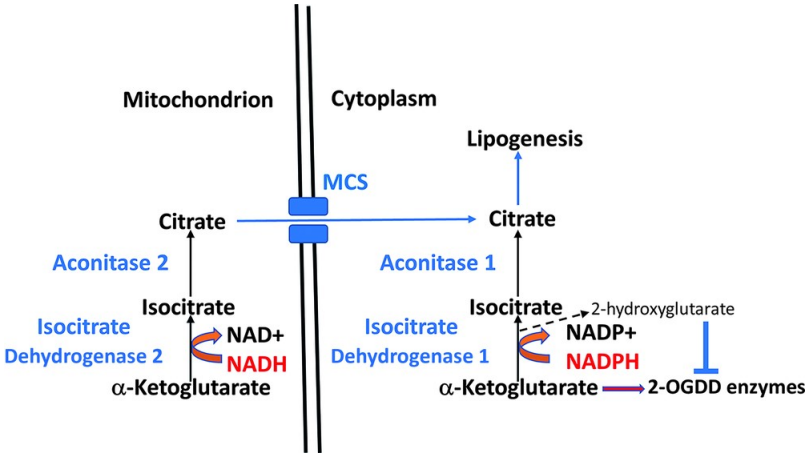

FIGURE 7.3 **The formation of citrate from α-ketoglutarate in either the mitochondrion or the cytoplasm**. Specifics are discussed in the text.

The α-ketoglutarate (also called 2-oxoglutarate) derived from either glutamine or glucose is important in a number of other cellular functions that may be important for viral infection.[90] For example, α-ketoglutarate is a co-substrate for 2-oxoglutarate-dependent dioxygenases (2-OGDDs).[67] The 2-OGDDs are a group of enzymes that catalyze hydroxylation reactions through the conversion of α-ketoglutarate to succinate. The 2-OGDDs have many substrates, including proteins, nucleic acids, and lipids. Importantly, α-ketoglutarate is a key modulator of the hypoxic response. Specifically, the prolyl-hydroxylases that regulate hypoxia-inducing factor-1α (HIF-1α), through proline hydroxylation, are 2-OGDD enzymes.[40] Further, α-ketoglutarate is a required substrate of some chromatin-modifying enzymes; thus, it has a role in epigenetic controls.[81]

Under some physiological conditions, isocitrate dehydrogenase can form 2-hydroxyglutarate from α-ketoglutarate (Fig. 7.3). Its formation is generally well controlled; however, it can accumulate to higher levels when there is a gain-of-function mutation in either the cytoplasmic or mitochondrial forms of isocitrate dehydrogenase.[30,173] The important point is that 2-hydroxyglutarate inhibits the 2-OGDD enzymes and thus functions in opposition to α-ketoglutarate for these enzymes. The production of 2-hydroxyglutarate has not been analyzed during viral infections, but one can imagine that for the life cycle of some viruses its production could be important, for example in altering epigenetic controls.

Other Functions of TCA Cycle Intermediates that May be Utilized by Viral Infections

We have discussed at length the non-TCA cycle functions of acetyl-CoA and α-ketoglutarate and how these should be considered as potential targets for manipulation during a viral infection. Other TCA metabolites also have non-TCA cycle functions.[90] Of note, increasing evidence suggests that the TCA cycle intermediates play crucial roles in regulating cellular immunity.[20] This would certainly be an aspect of the functions of TCA cycle intermediates that could be very useful for many viral infections.

We will look at a few examples of non-TCA cycle functions of TCA cycle intermediates that may affect viral infections:

- *Succinate*. Succinate plays an important role in inflammatory, hypoxic, and metabolic signaling[104]; it also has been reported to affect innate immunity.[90,150] Succinate can inhibit 2-OGDD enzymes, as it turns out it is an inhibitor of the proline hydroxylases that control HIF-1α levels, thus promoting the accumulation of HIF-1α in the presence of oxygen, also known as pseudohypoxia.[133] The control of HIF-1α is important in many viral infections.[80]
- *Fumarate*. Fumarate can function as an immunomodulator by controlling chromatin modifications and regulating protein succination.[4]
- *Malate*. Malate can be shuttled from the mitochondria to the cytosol where malic enzyme can use NADP+ to convert it to oxaloacetate and NADPH.[186] NADPH is an important reducing equivalent used in lipogenesis.

There are many more functions of TCA intermediates that we could discuss.[90] However, the important point is that the TCA cycle provides not only a means to make ATP but also valuable intermediates that have many other functions that could either support or inhibit a viral infection depending on the life cycle of the specific virus. They should be considered in any host-virus interaction study involving metabolism.

The Mitochondrion

In discussing the ETC and TCA cycle, we should stop to consider the organelle that contains them, the mitochondrion. Many viral studies have shown morphological (fission, fusion, expansion) and functional changes in the mitochondria during infection.[78,157] These studies often relate to the well-studied role of the mitochondria in apoptosis and immunity. Apoptotic signals that target mitochondria can affect morphology and increase permeability of the mitochondrial membrane, causing apoptotic effectors to leak out. Many viruses induce mechanisms to inhibit apoptosis. Cytomegaloviruses are a good example, encoding a mitochondria-localized inhibitor of apoptosis (vMIA), capable of suppressing apoptosis induced by diverse stimuli.[43,88] Additionally, under cell stress conditions, some of which may be induced by viral infection, the mitochondrial membrane may be disrupted and release the TCA cycle intermediates into the cytosol, which can activate mechanisms that impact cellular immunity.[20] All of these functions that affect mitochondria would be important for viral infections and may be manipulated during infection, either positively or negatively, depending on the virus's specific life cycle.

The effects of viral infection on the TCA cycle and ETC have been less studied; however, the examinations of HCMV metabolomics and glutaminolysis discussed above suggest that the bioenergetics of the mitochondrion are likely to be of key importance for many viral infections. Indeed, mitochondrial morphology changes during HCMV and KSHV infection, and disruption of ETC activity is quite deleterious to HCMV replication.[8,26,27,59] During HCMV infection the transcription and protein levels of ETC and ATP synthase proteins are increased[27,102]; similar

results have been reported with KSHV.[59] One must remember that many of the ETC proteins are encoded in the mitochondrial DNA. Transcription of the DNA requires a mitochondrial-specific RNA polymerase, which is quite different from nuclear RNAs polymerases, as it is believed to have evolved from a proteobacterium.[138] Likewise, translation requires mitochondrial-specific ribosomes and tRNAs.[48] Thus, it is interesting to consider that a virus may have evolved special mechanisms to interact with mitochondrial transcription and/or translation. However, the effects may be more indirect, possibly a general cellular response to many viral infections, or the result of stress responses induced by the infection. In any case, this will be an interesting area to watch as we learn more about the effects of viruses on cell metabolism and bioenergetics.

One last point about viral effects on mitochondria: it has been shown that an HCMV noncoding RNA (ncRNAβ2.7) stabilizes mitochondrial membrane potential, which assures ATP production during infection.[124] Up until now, we have not considered the potential effects that virally encoded lincRNAs, lncRNAs, and microRNAs may have on cellular metabolism because so little is known. It is known that cellular microRNAs affect metabolism,[164] often through their well-known controls of gene expression. Viral noncoding and microRNAs may do the same, but it is intriguing to consider that they may do more, consider the possibility that they have roles in allosteric regulation, effects on mitochondrial membranes, mitochondrial structure or function, or effects on transporter functions, for instance for glucose or glutamine transport. These are all important considerations as virologists delve deeper into the complex relationships between viruses and the metabolism of the cells they infect.

Lipid Metabolism

Lipids help form membranes and are a form of energy storage. Some lipids have more specialized functions such as being hormones or acting as signaling mediators. Lipids are structurally and chemically diverse. They can have different backbones, headgroups, and tails. Mammalian cells may have over 5000 different lipids.[55,136] They are organized into several classes, typically according to their backbone and headgroup structures. For example, glycerophospholipids contain a glycerol backbone, one of several types of phosphate-containing headgroups, and one to two tails of varying lengths and double-bond content while other lipids, including sterols, contain 5- and 6-carbon ring structures instead of a glycerol backbone. All lipids within a class are synthesized by the same pathways, which means a single enzyme in lipid synthesis may have several dozen to >100 products. This is different from many of the metabolic pathways that we have discussed where an enzyme may have only one product.

In general, lipids are synthesized from small metabolites, such as acetyl-CoA and amino acids. Fatty acids, cholesterol, and other sterols are made from acetyl-CoA. Most of the glycerol in the backbone is derived from DHAP redirected from glycolysis (Fig. 7.1). ATP provides the energy to make the fatty acids and attach them to the lipid backbone. It is noteworthy that CTP is also consumed during lipid synthesis. CTP is integral to making phospholipids via two general mechanisms: CTP is reacted with the metabolite that will become the head (eg, formation of CDP-choline or CDP-ethanolamine) or CDP is first conjugated to the backbone (eg, formation of CDP-diacylglyceride) (Fig. 7.4).

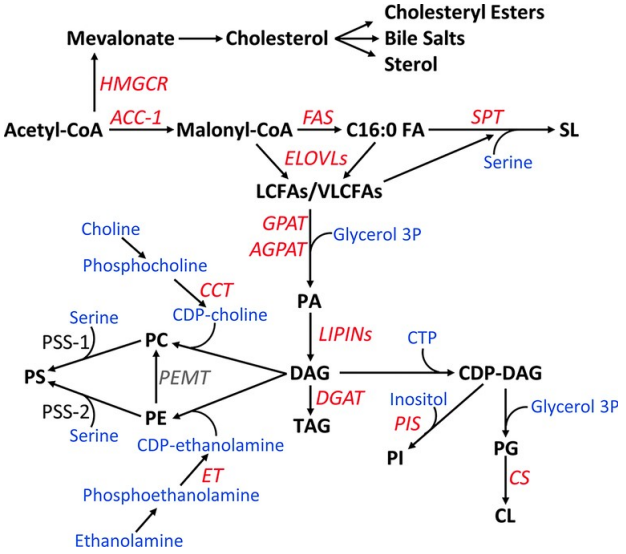

FIGURE 7.4 **Lipid Synthesis**. The *de novo* synthesis of lipids via different pathways starts with a common precursor: acetyl-CoA. Acetyl-CoA can be converted to mevalonate through the action of HMG-CoA reductase (HMGCR). This is the rate-limiting step of cholesterol synthesis. Mevalonate is the precursor to cholesterol. Cholesterol can be further metabolized to cholesteryl esters, bile salts, and sterols. Alternatively, acetyl-CoA can be converted to malonyl-CoA by acetyl-CoA carboxylase (ACC-1). This is the rate-limiting step in fatty acid synthesis. Fatty acid synthase (FAS) builds palmitic acid (C16:0) using carbon units from acetyl-CoA and malonyl-CoA. Fatty acids (FA) can be further elongated through the action of elongases (ELOVLs) to generate long-chain FAs (LCFAs) and very long-chain FAs (VLCFAs). FA can be used to generate sphingolipids (SL) via serine palmitoyl transferase (SPT). FAs can also be directed to acylglycerol lipid synthesis. Glycerol-3-phosphate acyltransferase (GPAT) and 1-acylglycerol-3-phosphate acyltransferase (AGPAT) add two FA tails to the glycerol 3-phosphate (glycerol 3P) to generate the simple phospholipid, phosphatidic acid (PA). LIPIN phosphatases (LIPINs) converted PA to diacylglycerol (DAG). A third fatty acid tail can be added to DAG by diglyceride acyltransferase (DGAT) to generate triacylglycerol (TAG). Alternatively, DAG can be converted to phosphatidylcholine (PC) via a *de novo* synthesis pathway involving choline metabolism. The rate-limiting reaction in PC synthesis is performed by CTP:phosphocholine cytidylyltransferase (CCT). Similar to PC synthesis, phosphatidylethanolamine (PE) is made from a pathway involving ethanolamine metabolism. In this case, CTP:phosphoethanolamine cytidylyltransferase (ET) is the rate-limiting enzyme. PC and PE can be converted to phosphatidylserine (PS) by the action of PS synthase-1 and -2 (PSS-1, PSS-2). Other phospholipids are made using CDP-DAG as a precursor. Phosphatidylinositol (PI) is made from CDP-DAG and inositol by PI synthase (PIS). CDP-DAG is converted to phosphatidylglycerol (PG) in multiple steps that consume glycerol 3P. In a final step, PG acts as a precursor for the synthesis of cardiolipins (CL) by CL synthase (CS).

Lipid synthesis occurs at several locations within a cell. Fatty acid synthase is in the cytosol, while fatty acid elongation and several other pathways of lipid synthesis occur in the ER. The Golgi, mitochondria, and peroxisomes serve as additional sites for lipid metabolism. Subcellular localization of enzymes involved in lipid metabolism can regulate activity through a pathway. As we will discuss later, viruses can take advantage of the regulation of lipid metabolism at several levels, including the subcellular localization of enzymes. We mentioned earlier that related viruses have divergent effects on host glycolytic and nucleotide metabolism. This is true for lipid metabolism as well. For example, flaviviruses are metabolically divergent as evident by the depletion of ceramides prior to infection suppresses West Nile virus (WNV) replication but enhances dengue virus (DENV) replication.[3] The metabolism of the major classes of lipids is defined, but the pathways involved in the synthesis of some lipids are poorly understood or only predicted. For this chapter, we will focus on what is known regarding the metabolic pathways for the major classes of lipids.

FA Synthesis, Elongation, and Desaturation

Fatty acids are built from acetyl-CoA and malonyl-CoA (Fig. 7.4). Acetyl-CoA carboxylase (ACC) adds a carbon from bicarbonate to acetyl-CoA to generate three-carbon malonyl-CoA in an ATP-dependent reaction. Fatty acid synthase (FAS) extends a growing fatty acid chain by adding two carbons from malonyl-CoA. The two carbons incorporated into the hydrocarbon are from the “acetate” unit of acetyl-CoA, while the carbon from bicarbonate is lost. As discussed earlier the two carbons that become part of the fatty acid chain are most often derived from glucose, acetate, or glutamine. FAS produces fatty acid chains 16 carbons (C16) in length. The newly produced fatty acid can be directed towards lipid synthesis to be incorporated as a tail in a lipid or can be further processed by acting as a substrate for fatty acid elongases or desaturases. Elongases extend fatty acid chains, again using malonyl-CoA as a carbon donor, to generate chains longer than 16 carbons. Like FAS, elongases added the new carbons to the carboxylic end of the growing chain. Each cycle where two carbons are added requires the reduction of two double bonds. Both FAS and elongases use NADPH as a cofactor to reduce the double bonds. Fatty acid desaturases catalyze the formation of carbon-carbon double bonds in the chain. Elongated and desaturated fatty acid chains can be used to form the tails of lipids. Once a chain becomes part of a lipid, elongases can no longer elongate it because the carboxylic end is no longer accessible since that is the end that is bound to the lipid glycerol backbone.

Virus infection creates a demand for fatty acids that is beyond the normal cellular demand. Several viruses are known to alter fatty acid metabolism. HCMV increases the flow of carbons from glycolysis and acetate through ACC, FAS, and elongation.[103,119,181,190–192] For this virus, the newly synthesized and elongated fatty acid chains are incorporated into lipids that function in the virus envelope and may likely support virus replication in other ways.[119] Vaccinia virus replication also requires *de novo* fatty acid synthesis to occur in the cytoplasm, but in contrast to HCMV fatty acids are imported into the mitochondria for breakdown for energy.[49] The synthesis and utilization of fatty acids in vaccinia virus may reflect the need for metabolic partitioning in organelles due to the nature of vaccinia's replication in the cytoplasm with little nuclear requirement. Viruses use several mechanisms to alter the activity in fatty acid metabolism. HCMV activates transcription factors that increase the expression of ACC, FAS, elongases, and desaturases.[119,143,190] Flaviviruses provide an example of how infection promotes the synthesis and desaturation of fatty acids and alters enzyme subcellular localization. Both FAS and stearoyl-CoA desaturase support dengue virus replication after being recruited to sites of replication.[51,57]

Fatty acids are not only directed toward lipid synthesis. Fatty acids can be used to modify proteins, both host and viral proteins. Saturated fatty acids with 14-carbon and 16-carbon chains are covalently attached to proteins via processes called myristoylation and palmitoylation. These types of modifications help viral proteins perform their functions. For example, HIV-1 Gag is myristoylated to increase its affinity for the plasma membrane for assembly, and palmitoylation directs glycoproteins to sites in the plasma membrane where the assembled immature virus will bud.[126,149] This example demonstrates how a virus can take advantage of the diversity in fatty acid modification performed by the host to coordinate its assembly and release.

Polyunsaturated fatty acids—ones that contain multiple double-bonds in the hydrocarbon chain—can be further metabolized via oxygenation to form a large class of bioactive molecules termed eicosanoids. Their synthesis can occur via three major pathways: cyclooxygenase (COX), lipoxygenase, and epoxygenase. The major precursor in these pathways is the 20-carbon polyunsaturated arachidonic fatty acid that has four double bonds (C20:4). Oxygenation by COX enzymes leads to prostaglandins and thromboxanes, which can be proinflammatory. Lipoxygenase products, including leukotrienes, and epoxygenase products also have potent proinflammatory signaling properties. Unsurprisingly, these proinflammatory responses can be detrimental to the virus and host if inflammation is severe.[22] However, for HCMV, COX-2 activity promotes virus replication,[194] demonstrating that viruses can use arachidonic acid metabolites to their advantage. Other polyunsaturated fatty acids can be oxygenated to form anti-inflammatory metabolites. These include eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6) that are used to generate bioactive molecules termed resolvins. Treatment with a resolvin promoted recovery from an ocular HSV-1 infection in an animal mode, suggesting that their anti-inflammatory properties can positively impact the outcome of infection.[121] This is a promising observation; however, anti-inflammatory fatty acid metabolites have not been well studied to understand if they have a direct impact on viral replication and if this impacts the outcome of virus infection more broadly.

Acylglycerol Lipids

Acylglycerol lipids contain a glycerol backbone to which one to three tails are esterified (Fig. 7.5). This class of lipids includes phospholipids that are major constituents in biological membranes and triacylglycerols, which efficiently store energy. Acylglycerol lipids are synthesized via pathways that have a shared beginning that starts with the generation of glycerol 3-phosphate from glycerol or DHAP (see Glycolysis).

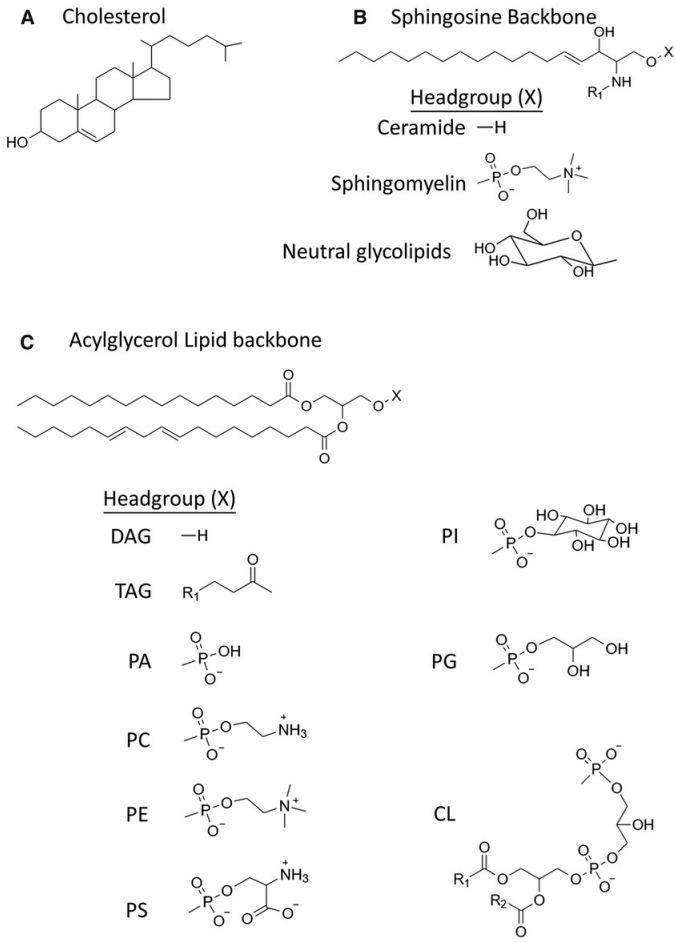

FIGURE 7.5 **Structure of the Major Classes of Lipids**. **A**. The structure of cholesterol is shown. **B**. The structures of sphingolipids are shown. Sphingolipids are characterized by a shared sphingosine backbone. Sphingolipids have an amide-linked fatty acid tail, typically saturated or monounsaturated (denoted as R1). They have variable headgroups (denoted as "X"). Ceramide is the most basic sphingolipid and "X" is a hydrogen (–H). Sphingomyelin has a phosphocholine head and glycolipids contain complex saccharides. **C**. The structures of acylglycerol lipids are shown. Glycerol functions as the backbone and two fatty acyl tails are attached via ester bonds. The composition of each fatty acid tail can vary in both carbon length and degree of saturation (shown is a saturated fatty acyl tail with no double-bonds, and a polyunsaturated tail with two double-bonds). "X" denotes the variable head-group. The simplest acylglycerol lipids is diacylglycerol (DAG), where "X" is a -H. Instead of a headgroup at "X", triacylglycerol (TAG) lipids contain a third fatty acyl tail denoted as R1. Phosphatic acid (PA) is the simplest acylglycerolphospholipid (ie, phospholipids) and only contains a phosphate headgroup. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) contain a phosphocholine, phosphoethanolamine, and phosphoserine as headgroups. Phosphatidylinositol (PI) and phosphatidylglycerol (PG) contain phosphoinositol and phosphoglycerol as headgroups. Cardiolipids (CL) are complex. Structurally, CL are two PA lipids connected together via a glycerol. This figure was made with the help of Ian Kline.

The attachment (ie, esterification) of fatty acids to the hydroxyl positions of glycerol 3-phosphate generates the first lipid in acylglycerol synthesis, phosphatidic acid (PA) (Fig. 7.4). The phosphate in PA lipids commonly comes from glycolysis via DHAP and is not further modified, making PA the simplest acylglycerolphospholipid (commonly called "phospholipid") (Fig. 7.5). PA is an early intermediate in the synthesis of other acylglycerols. Relative to other phospholipids, PA concentration in cells is low and most of it is further metabolized to other acylglycerol lipids. The next step in acylglycerol lipid synthesis is the formation of diacylglycerols (DAGs). DAGs are made by removing the phosphate from PAs through the action of lipin phosphatases. DAGs form a hub in acylglycerol synthesis. DAGs can be directly converted to triacylglycerol (TAG) lipids or some phospholipids (Fig. 7.4). Alternatively, DAGs can be activated to generate CDP-DAG for further metabolism to other phospholipids in a process that consumes CTP (Fig. 7.4). In addition to forming a metabolic hub, DAGs function as mediators of lipid signaling. Phospholipase cleavage of PIPs release DAG messengers that activate protein kinase C (PKC). The role of DAG signaling in virus infection has not been well studied, even though viruses exploit PKC to support replication.[97]

Triacylglycerols (TAGs) are formed following the addition of a third fatty acyl tail to DAGs (Fig. 7.4). Each of the three fatty acyl tails of TAGs can be oxidized to generate ATP via the TCA cycle as we will discuss in detail later. TAGs are a more efficient form of energy than carbohydrates and much of the energy stored in the body is in the form of TAGs in lipid droplets. TAGs are generated when energy needs of cells are low and consumed when demands for energy are high. The replication of some viruses stimulates the synthesis of TAG.[182] On the other hand, other viruses, like dengue virus, consume TAGs to generate energy.[58] Dengue virus stimulates the degradation of lipid droplets through autophagy; however, the energy generated via TAG breakdown and the TCA cycle may not be enough to sustain infected cells since the overall levels of ATP drop.[35]

Instead of being used for TAG synthesis, DAGs can be directed towards phospholipids. Synthesis of phosphatidylethanolamine (PE) lipids occurs via the transfer of a phosphoethanolamine group from CDP-ethanolamine to DAG (Fig. 7.4). The CDP-ethanolamine is formed from CTP and phosphoethanolamine in a reaction that "activates" CDP-ethanolamine to enable the transfer of the phosphoethanolamine group to DAG.

The final reaction produces PE and CMP. PEs are one of the most abundant phospholipids in many biological membranes and impart important biophysical properties. As such, it is unsurprising that PEs are enriched in enveloped viruses.[82,89,151] Moreover, PEs are necessary to form subcellular compartments that act as the replication site for positive-stranded RNA viruses.[183,184] It is likely that PEs are found in many membranes or membranous structures that are remodeled by viruses.

Phosphatidylcholines (PCs) are the most abundant phospholipids in many cellular membranes and can be made via multiple routes. In most tissues, PCs are synthesized via *de novo* synthesis, which involves adding a phosphocholine group to DAG, in a pathway that is analogous to PE synthesis (Fig. 7.4). In this pathway, the phosphocholine is donated by CDP-choline, which is generated from CTP and choline. Alternatively, PCs can also be generated from PE through the addition of three methyl groups to the ethanolamine headgroup to form a choline headgroup. The methylation is performed by phosphatidylethanolamine n-methyltransferase (PEMT) and occurs in the liver, fat, and a few other tissues.[28,34,175] It is unknown if animal viruses promote the PEMT pathway. Yeast and most plants are unable to methylate PE to PC. Instead, plants generate phosphocholine that can be incorporated into PCs by repeated methylation of the phosphoethanolamine precursor. This process of PC synthesis is targeted by plant viruses. Brome mosaic virus protein 1a recruits enzymes in PC synthesis to viral replication sites.[193] This finding further highlights that synthesizing lipids at sites where they can support virus replication—as opposed to transporting them to these sites—benefits the virus. Other RNA viruses, including HCV and poliovirus, may use a similar strategy of relocating lipid synthesis enzymes to replication sites.[193] It is unknown if negative-stranded RNA or DNA viruses relocalize enzymes in PC synthesis to make these lipids at sites of virus replication and assembly. However, since PCs are abundant in enveloped viruses it is possible that other viruses recruit PC synthesis enzymes to make PCs at the site of envelopment.

In mammals, phosphatidylserine (PS) lipids are made from PE or PC via head-group exchange where serine replaces ethanolamine or choline (Fig. 7.4). In yeast and plants, PS is synthesized from CDP-DAG. PS is found in several membranes and has important signaling functions, particularly in apoptosis. PS lipids are sequestered on the intracellular side of the plasma membrane in healthy cells. Their externalization during apoptosis stimulates macrophage phagocytosis. PS lipids contribute to apoptotic clearance, which can be triggered by viral infection. Additionally, viruses that infect macrophages can take advantage of their ability to recognize PS lipids. Macrophages express TIM and TAM family receptors to interact with PS. These receptors can interact with PS lipids that are incorporated in the outer leaflet of the envelope of flaviviruses.[95] This finding demonstrates that flaviviruses can take advantage of apoptotic signaling without inducing apoptosis through the inherent function of PS lipids and TIM/TAM receptors.

Next, we will focus on phospholipids made from the CDP-DAG precursor. Phosphatidylinositols (PIs), like PS, are a minor constituent of the inner leaflet of the plasma membrane. PI species are synthesized by the condensation of inositol with CDP-DAGs (Fig. 7.4). Phosphorylation of the inositol headgroup generates phosphatidylinositol phosphate (PIP). The phosphorylation can occur at several locations on the PI head resulting in several forms of PIPs, which are important mediators of signaling. Signal transduction at the plasma membrane activates phospholipase C to cleave PIP into DAG and an inositol phosphate (IP). As discussed earlier, DAGs activate protein kinase C and IP acts as a secondary messenger by activating Ca^{2+}-dependent enzymes. Like PI, phosphatidylglycerol (PG) lipids are synthesized from CDP-DAG (Fig. 7.4). In this case, glycerol 3-phosphate is added to CDP-DAG resulting in a PG-3-phosphate. In the final step, the phosphate is removed by a phosphatase resulting in the PG product (Fig. 7.5). PG lipids can be further metabolized to cardiolipins (CLs) using CDP-DAG as a substrate (Fig. 7.4). Structurally, CLs are complex and are essentially two phospholipids linked together through a third glycerol backbone (Fig. 7.5). CLs are enriched in the inner mitochondrial membrane. Given their importance to mitochondrial function, CLs are likely essential to the metabolic activity of viral infected cells.

Mevalonate and Cholesterol Metabolism

Cholesterol is one of the most studied lipids, including research into understanding its functions in viral infections. Cholesterol contains 27 carbons, each is derived from acetyl-CoA (Fig. 7.5). The rate-controlling enzyme of cholesterol synthesis is HMG-CoA reductase, which produces mevalonate (Fig. 7.4). Mevalonate is phosphorylated and decarboxylated to generate a 5-carbon activated isoprene. Successive condensation of 5-carbon isoprenes results in a 30-carbon intermediate that is oxygenated, decarboxylated, and reduced to yield cholesterol. As discussed later, viruses may utilize several mechanisms to alter cholesterol homeostasis. In membranes, cholesterol can enhance fluidity, reduce permeability, strengthen lipid bilayers, and assist in raft generation. Cholesterol is integrated into the envelope of diverse viruses (eg, influenza, herpesviruses, and others).[17,21,50,89,146] Proper cholesterol content in the host membrane and virus envelope is important for entry and virus infectivity.[50,61,142,146] Cholesterol is important for vesicle trafficking that is necessary for virus replication and the formation of RNA virus replication organelles.[65,107,145,176] DNA virus replication and production of infectious progeny also require cholesterol.[75,180] Cholesterol may support virus replication by being further metabolized to cholesteryl esters or bile acids.

A fatty acid tail can be attached to cholesterol at the hydroxyl position, generating a cholesteryl ester. Hepatitis C virus infection increases cholesteryl ester synthesis to enhance virus assembly demonstrating that cholesterol esterification is necessary for the replication of some viruses.[79,123] Alternatively, cholesterol can be metabolized to form other sterols or bile acids. Recently, bile acid binding to norovirus capsid was found to induce a structural shift in the shell that enhances receptor binding and aids escape from antibody recognition.[177] These observations demonstrate that cholesterol metabolism and its downstream metabolites can perform diverse and essential functions in virus infection.

In addition to being a precursor to cholesterol, mevalonate can alternatively be metabolized to form farnesyl and geranylgeranyl groups. These 15- and 20-carbon lipid structures can be attached to a cysteine residue of a protein in a process called prenylation. These posttranslation modifications increase the hydrophobicity of the target protein and are essential for the function of some proteins involved in signaling, including Rab family members. Geranylgeranyltransferase II (GGT-II) is increased by Epstein-Barr virus (EBV) infection, and its activity is essential to EBV-mediated B-cell growth.[171] Prenylation of host proteins is also important for Hepatitis C virus replication.[169] Although prenylation of viral proteins is not well studied, it is possible and may be essential to the function of some viral proteins.[110]

Sphingolipids

Sphingolipids (SLs) are primarily found on the outer leaflet of the plasma membrane. They are made through the condensation of a fatty acyl-CoA and serine through the action of serine palmitoyl transferase (SPT). SPT can, in some cases, utilize other amino acids in addition to serine, providing further SL diversity. At least two SLs, ceramide and sphingosine 1-phosphate (S1P), act as signaling messengers. Determining the importance of SL to viruses can be complicated since SL has multiple functions. Several extracellular signals or stimuli activate SL signaling,

including immune-related TNFα, IL-1α, IL-1β, and IFNγ factors. Since SL signaling can modulate immune-related factors, regulations of SL metabolism could be a mechanism viruses may use to favor infection. SLs are enriched in microdomains of plasma membranes and in the envelope of some viruses, suggesting that SLs participate in the assembly and release of enveloped viruses.[13] SLs interact with HIV gp120 and gp41 glycoproteins to assist entry.[54] SLs are also necessary for nonenveloped virus entry suggesting that the infection of some viruses is dependent on SLs enriched on the outer leaflet of the plasma membrane.[46,85] Furthermore, SL-dependent entry has also been noted for other enveloped viruses, suggesting that SLs may support virus entry by functioning within the viral envelope membrane or host plasma membrane.[54,166] It may be the case, as demonstrated by measles virus, that SLs are important for virus infection by functioning in the viral or host membrane and through SL-mediated host signaling.[166]

Discovery of Novel Lipids

It is estimated that only half of the lipids in cells can be identified using some of the most extensive lipidomic methods.[136] Just now, the large diversity of lipids is being revealed. There is a growing appreciation for new biological functions and physiological roles provided by newly discovered lipids. Like proteins that are posttranslationally modified, lipids too can be modified. Lipids can be oxygenated or nitrated. A free fatty acid can be modified with another free fatty acid. For example, a palmitic acid (C16:0) can be esterified to a stearic acid (C18:0) forming branched chain fatty acids.[187] Since these modified lipids regulate both metabolism and inflammation,[187] they may be targeted for synthesis or degradation by viruses.

As we discussed earlier, amino acids are important metabolites in the synthesis of lipid heads. In addition to being incorporated into the head of lipids, amino acids can be added to the methyl end of a fatty acid tail. In this case, the hydrocarbons from branched chain amino acids are added to the methyl end of fatty acids generating branched fatty acids with an even or odd number of carbons.[168] This happens in adipose tissue where lipogenesis activity is high. Since many viruses promote lipogenesis, they may increase the production of these branched fatty acids. HCMV infection promotes fatty acid elongation leading to the production of lipids with very long chain tails that are not observed using most standard lipidomic approaches.[119,181,182] As analytical and bioinformatic methods are developed to identify currently unknown lipids, we will likely see that viruses can have more profound effects on lipid metabolism and the host lipidome than we currently appreciate. Since viruses can shift metabolism in ways that we still do not understand, it may be possible that infection will increase the production of many unknown lipids. Continued lipidomic studies of virus infection may lead the way to identifying unknown lipids and virology-focused studies may reveal functions of lipids that have yet been discovered.

The Fate of Lipids and Their Catabolism

Both lipid synthesis and degradation can support virus replication. All lipids are catabolized via similar mechanisms, but specific types of lipids are broken down by different pathways and enzymes. Lipids are oxidized for energy, degraded to repurpose their heads or tails, or redirected to provide additional functions. Fatty acids are broken down in the mitochondria to generate energy. Fatty acids are transported into the mitochondria through the carnitine shuttle (Fig. 7.2). First, a carnitine group is added to the carboxyl end of a fatty acid by carnitine acyltransferase I (CPT-I). This is the rate-limiting step of fatty acid β-oxidation. Once in the mitochondrial matrix, the carnitine group is removed, and the fatty acid is degraded from the carboxyl end. If the fatty acid has an even number of carbons, then it is completely oxidized through multiple cycles that remove 2-carbon per cycle to generate acetyl-CoA. The acetyl-CoA is further consumed in the TCA cycle to generate ATP. If the chain has an odd number of carbons, then the final products are acetyl-CoA and 3-carbon propionyl-CoA that requires further processing for the conversion to succinyl-CoA, a TCA cycle intermediate (Fig. 7.2). Branched chain and very-long-chain fatty acids are oxidized in peroxisomes in an analogous process that breaks down the fatty acid chain by 2-carbons per cycle. Viruses can stimulate lipid breakdown to generate energy, as discussed earlier for dengue virus.[35] Additionally, virus infection can alter the activity of lipases that break down lipids. Coronaviruses stimulate a phospholipase that breakdowns PE by cleaving one tail to liberate it as a free fatty acid. The other tail remains attached to the PE head, creating a one-tailed lysoPE.[101,185] Inhibiting phospholipases reduces virus replication and pathogenicity for several viruses, including coronaviruses.[101,165]

Regulation of Lipid Metabolism

Like glycolysis and the TCA cycle, lipid metabolism is controlled through several coordinated mechanisms. The best-understood regulations of lipid metabolism pathways are fatty acid and cholesterol synthesis. Both fatty acid and cholesterol synthesis are regulated at several levels. The rate-limiting step of fatty acid synthesis is the production of malonyl-CoA by acetyl-CoA carboxylase (ACC-1). Since fatty acid synthesis is an energy expensive process, they are made when the energy demands of the cells are low. When energy demands of the cell are high, then fatty acid synthesis is disfavored. Since fatty acid synthesis and catabolism help balance the energy demands of a cell, their metabolism is regulated by AMPK, an energy sensor. When energy demands are high, AMPK phosphorylates ACC-1 to inactivate it. In its active form, ACC-1 multimerizes, but phosphorylated ACC-1 favors a monomeric state providing further loss of activity. ACC-1 is both allosterically activated and inhibited. Carbons from the TCA cycle are directed to fatty acid synthesis in the form of citrate. Citrate is shuttled to the cytosol to form acetyl-CoA and malonyl-CoA. Increased abundance of cytosolic citrate promotes ACC-1 activation. The product of fatty acid synthase, palmitoyl-CoA, acts as a feedback inhibitor to allosterically reduce ACC-1 activity.

Like fatty acid synthesis, making cholesterol is energy demanding. Cholesterol metabolism is tightly regulated through an elegant feedback mechanism. The expression of most genes that encode cholesterol synthesis enzymes, including the rate-limiting step catalyzed by HMG-CoA reductase, is transcriptionally controlled by sterol regulatory element–binding proteins (SREBPs). When cholesterol levels are sufficient, SREBPs are held in the ER membrane in an immature, inactive form. In the ER, SREBP is bound to SREBP cleavage-activating protein (SCAP), which interacts with insulin-induced gene 1 protein (INSIG). When cholesterol levels in the ER fall, INSIG releases the SCAP-SREBP complex allowing it to transit to the Golgi. Here, S1P and S2P protease cleave SREBPs releasing the mature, active transcription factor forms of SREBPs. There are three isoforms of SREBPs: −1a, −1c, and −2. In general, SREBP-1a stimulates the expression of genes encoding cholesterol and fatty acid synthesis, while SREBP-1c turns on fatty acid synthesis genes and SREBP-2 turns on cholesterol synthesis genes. SREBPs also regulate the expression of low-density lipoprotein (LDL) receptor (LDLR). LDLR binds to cholesterol-rich LDL particles to import them. Consuming LDL particles provide an inexpensive source of cholesterol.

As highlighted earlier, several viruses promote fatty acid and cholesterol metabolism. Again, HCMV provides a good example of the elaborate mechanisms viruses may use to regulate these lipid metabolic pathways. HCMV infection activates SREBPs leading to increased expression of

genes encoding ATP-citrate lyase, ACC-1, HMG-CoA reductase, and fatty acid synthase, and elongases.[119,143,181,190] Interestingly, HCMV infection activates AMPK causing an increase in the inactive phosphorylated form of ACC-1.[92,143,152] However, the overall metabolic activity of ACC-1 is increased to overcome this inhibition, in part by SREBP-dependent increased expression of ACC-1.[143] Consequently there is an increase in cholesterol levels in the early stage of infection, followed by an increase in fatty acid levels.[1,50,119] Further, HCMV infection reduces the ABCA1 cholesterol efflux transporter to retain intracellular cholesterol.[130] Notably, shortly after HCMV infection LDL related receptor 1 (LRP1), which decreases intracellular cholesterol, is upregulated as a possible host defense response to infection that the virus overcomes at a late stage in replication.[50]

In addition to SREBPs, lipid metabolism is transcriptionally regulated by liver X receptors (LXRs) and retinoid X receptors (RXRs). LXR and RXR restrict HIV-1 infection in activated CD4+ T cells by promoting cholesterol efflux via the ABCA1 transporter.[69] A similar mechanism may restrict HCV and Newcastle disease virus.[9,134] In contrast to promoting virus replication, LXR may have some antiviral potential in certain contexts. Interferon-mediated expression of LXR reduces murine herpesvirus 68 (MHV68) replication in primary macrophages by repressing the synthesis of lipids that support virus replication.[74]

Since many steps of lipogenesis occur in the ER, there is a significant connection between ER homeostasis and lipids. Stress and lipid metabolism have a complex relationship that is poorly understood but is important to nonalcoholic fatty liver disease, obesity, diabetes, and cancers. The relationship between stress and lipid metabolism can be exploited by viruses. HCMV uses the molecular mediators of ER stress to regulate metabolism to support virus replication.[68,192] Specifically, HCMV activates PKR-like ER kinase (PERK; also known as eukaryotic translation initiation factor 2-alpha kinase 3, EIF2AK3) to promote lipid synthesis, in part by increasing the levels of fatty acid elongase ELOVL7.[182,192] Interestingly, PERK is not required for HCMV-induced increases in ELOVL5.[182] The ability of PERK to regulate the differential expression of ELOVLs was unknown prior to this work and may also occur in conditions where stress promotes lipid synthesis. Furthermore, PERK may also regulate lipid synthesis by targeting the DAG hub between phospholipid synthesis and TAG synthesis. PERK may promote the flow of DAGs towards phospholipids instead of TAGs in HCMV-infected cells.[182] If true, this would be an example where studies of virus-host metabolism interactions increase our understanding of how stress responses regulate metabolism.

Considerations Regarding Metabolic Studies in Virology

At this point, we will turn our attention to a discussion of concepts a virologist should consider when studying metabolism. Many different types of experiments may lead a virologist to consider studying metabolism. Genomic, transcriptomic, and proteomic studies of virus-host interactions may suggest that infection reprograms metabolism. However, it is important to remember that metabolic conclusions must be reached using metabolic assays.

In regard to genomics, some viruses may contain genes that encode proteins with homology to known host metabolic enzymes. For example, HSV-1 encodes several proteins that can perform metabolic reactions, including deoxyuridine triphosphatase (dUTPase), thymidine kinase, and ribonucleotide reductase.[5,6,16,29,38,94,115,116] The enzymatic actions performed by these viral enzymes support pyrimidine synthesis required for DNA replication.[10] Although the viral dUPTase is not required for replication in cell culture[38], it is important for establishing a latent infection and reactivation in a mouse model.[120] While HSV-1 is an example where viral protein homology of host enzymes can faithfully predict metabolic activity that occurs in infected cells, other herpesviruses offer a cautionary tale. HCMV encodes enzymatically inactive homologs of HSV-1 uracil DNA glycosylase (UL114), ribonucleotide reductase (UL45), and dUTPase (UL72).[15,19,73,113,122] The fact that these homologs lack enzymatic activity demonstrates that these genes are conserved for nonmetabolic functions. Gamma-herpesviruses further encode a viral formyl-glycinamide-phosphoribosyl-amidotransferase (vFGARAT) that is a homolog of the cellular purine synthesis enzyme.[158,161] The vFGARATs lack enzymatic activity; instead they have evolved divergent functions to promote virus replication and pathogenesis.[41,158,161] As discussed earlier, virologists should be aware that any metabolic enzyme may have divergent or alternate functions that may be completely unrelated to metabolism.

Similar to genomic studies, transcriptomic and proteomic studies have limited predictive value for understanding metabolic activity. This is particularly true in conditions that perturb metabolism in ways that are not thoroughly investigated or not yet well understood, such as changes in metabolism triggered by virus infection. Systems-level analyses of model organisms demonstrate that measurements of enzyme abundance poorly predict regulation for many metabolic reactions.[52,111] Changes in metabolic flux can occur within minutes, while transcriptionally driven changes may require many minutes or several hours to change in response to metabolic stimuli. While the rate of some metabolic reactions is regulated by enzyme concentration, most enzymes are expressed in abundance and are regulated by the metabolite-substrate availability or allosteric regulation[52,111] (see the section on Allosteric Regulation).

Metabolomics and lipidomics provide a picture of how viruses may alter the host metabolic profile. These techniques offer a catalog of changes but do not describe the metabolic activity through a pathway. For example, if infection increases the abundance of citrate, this observation alone cannot lead to the conclusion that there is an increase in the activity of the TCA cycle or fatty acid synthesis. Supplementing metabolomic or lipidomic findings with measurements of metabolic activity is required to build a more complete picture of virus-host metabolism interactions. There are several approaches to experimentally define metabolic activity in a pathway. Measurements of extracellular acidification and glucose consumption can provide an understanding of glycolytic changes in response to infection.[27,31] Similarly, the oxygen consumption rate can be used as a measure of activity in oxidative-phosphorylation. These approaches led to the discovery that norovirus infection in macrophages increases ATP generated through glycolysis while slightly reducing mitochondrial production of ATP.[112] However, the best way to determine if a virus alters the flow of nutrients or the flux in additional metabolic pathways is through the use of metabolic tracers, typically isotopically labeled metabolites. In a 2008 paper, Munger et al used ^{13}C-labeled glucose and glutamine to determine the effects of HCMV infection on central carbon metabolism.[103] HCMV infection profoundly increased the metabolism of glucose, TCA activity, and the flow of carbons to fatty acid synthesis. This work demonstrated the power in metabolomics, isotopic tracers, and flux determination that has subsequently led to many additional findings. Afterward, it was demonstrated that HCMV also uses carbons derived from acetate to support lipid synthesis and virus replication using ^{13}C-acetate.[167] Additionally, these initial discoveries further led to the discovery that HCMV increases fatty acid elongation using ^{13}C-glucose and ^{13}C-acetate,[119,181] total lipid synthesis using ^{14}C-acetate,[190,192] UDP-sugar metabolism for glycosylation of viral proteins,[33] and many of the other findings regarding HCMV discussed in this chapter. Moreover, this work by Munger et al. provided a roadmap for studying other viruses.[93,148,163,170]

As discussed earlier, phosphoenolpyruvate carboxykinase (PEPCK) has nonmetabolic functions that may impact viruses. Several metabolites and enzymes similarly have functions outside of metabolism.[63] As another example related to glycolysis, phosphoglucose isomerase (PGI) functions in metabolism to convert glucose-6-phosphate (G6P) to fructose-6-phosphate (F6P). PGI also has a nonmetabolic function as an autocrine factor that promotes cell survival, migration, and proliferation.[174] Similarly, some metabolites have nonmetabolic functions. Kynurenine is a good example to highlight metabolic and non-metabolic functions of metabolites. Kynurenine is a metabolite in tryptophan degradation and an intermediate in NAD+ regeneration. Additionally, kynurenine is a ligand for the aryl hydrocarbon receptor (AhR) that acts as a transcription factor upon binding kynurenine. These nonmetabolic functions can impact the outcome of a virus infection and should be considered when studying metabolism in virology.[106,178] For those who want to further understand the noncanonical functions of metabolic enzymes, we recommend the review by Huangyang and Simon.[63]

Metabolism in the Host Fight Against Virus Infection

Much of this chapter focuses on the role of metabolism in the support of infection and virus replication, which reflects the focus of most studies in virus-host metabolism interactions. However, metabolism is also integral to the host antiviral response and can be used to suppress infection or virus replication. In recent years, the field of immunometabolism—the study of the role of metabolism in immunology—has grown into a mature field. Metabolism is essential in forming the immune response to cancers, self in autoimmune disorders, and pathogens including viruses. Rather than review the rapidly growing field of immunometabolism, we encourage any readers interested in learning more to read the following reviews regarding immunometabolism in general,[87] B cells,[125] T cells,[42] and myeloid cells.[162]

Furthermore, a few initial metabolic studies in virology have shifted our understanding of virus-host interactions to reveal that the host can use metabolism to limit virus-induced effects. A reduction in TCA activity and smaller nucleotide pools were associated with a growth arrest of B cells infected with EBV. In this case, metabolism creates a barrier to virus-induced cell immortalization.[53,93] Interventions to raise the metabolic barriers could provide benefits to reducing the oncogenic potential of EBV and other oncogenic viruses.[11] Herpesviruses provide an additional example where host metabolism is targeted to increase a barrier to virus replication. In response to HCMV infection, the host uses HIF-1α's metabolic regulatory activity to limit kynurenine levels.[178] Since kynurenine-mediated signaling promotes HCMV replication, HIF-1α suppression of its synthesis limits HCMV replication.[106,178] As more metabolic studies are performed in virology, it is likely that additional mechanisms the host uses to create barriers to infection and virus replication will be discovered.

Summary and Future Outlooks

We have attempted to provide reasons for the importance of metabolic studies in virology. Clearly, metabolism connects and interfaces with every function of the cell and vice versa. It is an ideal target for viruses to influence host cell functions and an ideal means for the host to fight back. Yet we have only scratched the surface of metabolism, glycolysis, TCA cycle, mitochondrial function, glutaminolysis, and lipid metabolism. There is so much more, so many more host metabolic and biosynthetic pathways with which viruses can interact.

Understanding the interactions between viral infections and host metabolism will provide many new avenues for the development of antiviral drugs through the inhibition of specific metabolic reactions or pathways. In the past, many have felt that metabolism was sacrosanct, that drugs targeting metabolism would be too deleterious. But this is not correct; there are many people who survive, albeit, with hardship, the loss of significant metabolic pathways due to mutations,[47] and cancer biology is showing how antimetabolic drugs may be effective cancer chemotherapy.[2] There is a great opportunity for antiviral drugs based on temporary metabolic interference; we just have to decipher and understand the intricate dance that goes on between the virus and host metabolism.

Future work in this field should strive to use models that best capture *in vivo* environments. For virus-host metabolism studies done in cell models, one should consider how the environment influences metabolism. Alterations in cell culture media, oxygen levels, confluency, cell-cycle and other physiological relevant conditions can have profound effects on both metabolism and virus replication. The natural history of the cell is also an important factor, especially in the case of studies performed in transformed or oncogenic cells where metabolism has already been altered to support proliferation regardless of the viral infection. These consideration are not unique to virology; they are challenging for all fields studying metabolism in health and disease. Moreover, additional work investigating how metabolic disease in the host may influence virus infection is much needed.[140] We hope that the rich history of virology may provide some advantages as the field continues to develop.

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REFERENCES

1. Abrahamsen LH, Clay MJ, Lyle JM, et al. The effects of cytomegalovirus infection on polar lipids and neutral lipids in cultured human cells. Intervirology. 1996;39(4):223-229.

2. Akins NS, Nielson TC, Le HV. Inhibition of glycolysis and glutaminolysis: an emerging drug discovery approach to combat cancer. Curr Top Med Chem. 2018;18(6):494-504.

3. Aktepe TE, Pham H, Mackenzie JM. Differential utilisation of ceramide during replication of the flaviviruses West Nile and dengue virus. Virology. 2015;484:241-250.

4. Arts RJ, Novakovic B, Ter Horst R, et al. Glutaminolysis and fumarate accumulation integrate immunometabolic and epigenetic programs in trained immunity. Cell Metab. 2016;24(6):807-819.

5. Baldo AM, McClure MA. Evolution and horizontal transfer of dUTPase-encoding genes in viruses and their hosts. J Virol. 1999;73(9):7710-7721.

6. Barker DE, Roizman B. Identification of three genes nonessential for growth in cell culture near the right terminus of the unique sequences of long component of herpes simplex virus 1. Virology. 1990;177(2):684-691.

7. Bauer DE, Hatzivassiliou G, Zhao F, Andreadis C, Thompson CB. ATP citrate lyase is an important component of cell growth and transformation. Oncogene. 2005;24(41):6314-6322.

8. Betsinger CN, Jankowski CSR, Hofstadter WA, et al. The human cytomegalovirus protein pUL13 targets mitochondrial cristae architecture to increase cellular respiration during infection. Proc Natl Acad Sci U S A. 2021;118(32):e2101675118.

9. Bocchetta S, Maillard P, Yamamoto M, et al. Up-regulation of the ATP-binding cassette transporter A1 inhibits hepatitis C virus infection. PLoS One. 2014;9(3):e92140.

10. Boehmer PE, Lehman IR. Herpes simplex virus DNA replication. Annu Rev Biochem. 1997;66:347-384.

11. Bonglack EN, Messinger JE, Cable JM, et al. Monocarboxylate transporter antagonism reveals metabolic vulnerabilities of viral-driven lymphomas. Proc Natl Acad Sci U S A. 2021;118(25):e2022495118.

12. Boodhoo N, Kamble N, Sharif S, Behboudi S. Glutaminolysis and glycolysis are essential for optimal replication of Marek's disease virus. J Virol. 2020;94(4):e01680-19.

13. Brugger B, Glass B, Haberkant P, Leibrecht I, Wieland FT, Krausslich HG. The HIV lipidome: a raft with an unusual composition. Proc Natl Acad Sci U S A. 2006;103(8):2641-2646.

14. Buchkovich NJ, Yu Y, Zampieri CA, Alwine JC. The TORrid affairs of viruses: effects of mammalian DNA viruses on the PI3K-Akt-mTOR signalling pathway. Nat Rev Microbiol. 2008;6(4):266-275.

15. Caposio P, Riera L, Hahn G, Landolfo S, Gribaudo G. Evidence that the human cytomegalovirus 46-kDa UL72 protein is not an active dUTPase but a late protein dispensable for replication in fibroblasts. Virology. 2004;325(2):264-276.

16. Caradonna S, Worrad D, Lirette R. Isolation of a herpes simplex virus cDNA encoding the DNA repair enzyme uracil-DNA glycosylase. J Virol. 1987;61(10):3040-3047.

17. Cervin M, Anderson R. Modulation of coronavirus-mediated cell fusion by homeostatic control of cholesterol and fatty acid metabolism. J Med Virol. 1991;35(2):142-149.

18. Chambers JW, Maguire TG, Alwine JC. Glutamine metabolism is essential for human cytomegalovirus infection. J Virol. 2010;84(4):1867-1873.

19. Chee MS, Bankier AT, Beck S, et al. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr Top Microbiol Immunol. 1990;154:125-169.

20. Choi I, Son H, Baek J-H. Tricarboxylic acid (TCA) cycle intermediates: regulators of immune responses. Life (Basel). 2021;11(1):69.

21. Chung CS, Huang CY, Chang W. Vaccinia virus penetration requires cholesterol and results in specific viral envelope proteins associated with lipid rafts. J Virol. 2005;79(3):1623-1634.

22. Cilloniz C, Pantin-Jackwood MJ, Ni C, et al. Lethal dissemination of H5N1 influenza virus is associated with dysregulation of inflammation and lipoxin signaling in a mouse model of infection. J Virol. 2010;84(15):7613-7624.

23. Clippinger AJ, Alwine JC. Dynein mediates the localization and activation of mTOR in normal and human cytomegalovirus-infected cells. Genes Dev. 2012;26(18): 2015-2026.

24. Clippinger AJ, Maguire TG, Alwine JC. The changing role of mTOR kinase in the maintenance of protein synthesis during human cytomegalovirus infection. J Virol. 2011;85(8):3930-3939.

25. Clippinger AJ, Maguire TG, Alwine JC. Human cytomegalovirus infection maintains mTOR activity and its perinuclear localization during amino acid deprivation. J Virol. 2011;85(18):9369-9376.

26. Combs JA, Monk CH, Harrison MAA, et al. Inhibiting cytomegalovirus replication through targeting the host electron transport chain. Antiviral Res. 2021;194:105159.

27. Combs JA, Norton EB, Saifudeen ZR, et al. Human cytomegalovirus alters host cell mitochondrial function during acute infection. J Virol. 2020;94(2):e01183-19.

28. Crews FT, Hirata F, Axelrod J. Identification and properties of methyltransferases that synthesize phosphatidylcholine in rat brain synaptosomes. J Neurochem. 1980;34(6):1491-1498.

29. Daikoku T, Yamamoto N, Maeno K, Nishiyama Y. Role of viral ribonucleotide reductase in the increase of dTTP pool size in herpes simplex virus-infected Vero cells. J Gen Virol. 1991;72(Pt 6):1441-1444.

30. Dang L, White DW, Gross S, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature. 2009;462(7274):739-744.

31. Delgado T, Carroll PA, Punjabi AS, Margineantu D, Hockenbery DM, Lagunoff M. Induction of the Warburg effect by Kaposi's sarcoma herpesvirus is required for the maintenance of latently infected endothelial cells. Proc Natl Acad Sci U S A. 2010;107(23):10696-10701.

32. Delgado T, Sanchez EL, Camarda R, Lagunoff M. Global metabolic profiling of infection by an oncogenic virus: KSHV induces and requires lipogenesis for survival of latent infection. PLoS Pathog. 2012;8(8):e1002866.

33. DeVito SR, Ortiz-Riano E, Martinez-Sobrido L, Munger J. Cytomegalovirus-mediated activation of pyrimidine biosynthesis drives UDP-sugar synthesis to support viral protein glycosylation. Proc Natl Acad Sci U S A. 2014;111(50):18019-18024.

34. Dudeja PK, Foster ES, Brasitus TA. Synthesis of phosphatidylcholine by two distinct methyltransferases in rat colonic brush-border membranes: evidence for extrinsic and intrinsic membrane activities. Biochim Biophys Acta. 1986;875(3):493-500.

35. El-Bacha T, Midlej V, Pereira da Silva AP, et al. Mitochondrial and bioenergetic dysfunction in human hepatic cells infected with dengue 2 virus. Biochim Biophys Acta. 2007;1772(10):1158-1166.

36. Eniafe J, Jiang S. The functional roles of TCA cycle metabolites in cancer. Oncogene. 2021;40(19):3351-3363.

37. Faubert B, Li KY, Cai L, et al. Lactate metabolism in human lung tumors. Cell. 2017;171(2):358-371 e9.

38. Fisher FB, Preston VG. Isolation and characterisation of herpes simplex virus type 1 mutants which fail to induce dUTPase activity. Virology. 1986;148(1):190-197.

39. Fontaine KA, Camarda R, Lagunoff M. Vaccinia virus requires glutamine but not glucose for efficient replication. J Virol. 2014;88(8):4366-4374.

40. Frost J, Frost M, Batie M, Jiang H, Rocha S. Roles of HIF and 2-oxoglutarate-dependent dioxygenases in controlling gene expression in hypoxia. Cancers (Basel). 2021;13(2):350.

41. Full F, Jungnickl D, Reuter N, et al. Kaposi's sarcoma associated herpesvirus tegument protein ORF75 is essential for viral lytic replication and plays a critical role in the antagonization of ND10-instituted intrinsic immunity. PLoS Pathog. 2014;10(1):e1003863.

42. Geltink RIK, Kyle RL, Pearce EL. Unraveling the complex interplay between T cell metabolism and function. Annu Rev Immunol. 2018;36:461-488.

43. Goldmacher VS, Bartle LM, Skaletskaya A, et al. A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. Proc Natl Acad Sci U S A. 1999;96(22):12536-12541.

44. Goodwin CM, Xu S, Munger J. Stealing the keys to the kitchen: viral manipulation of the host cell metabolic network. Trends Microbiol. 2015;23(12):789-798.

45. Grady SL, Purdy JG, Rabinowitz JD, Shenk T. Argininosuccinate synthetase 1 depletion produces a metabolic state conducive to herpes simplex virus 1 infection. Proc Natl Acad Sci U S A. 2013;110(51):E5006-E5015.

46. Grassme H, Riehle A, Wilker B, Gulbins E. Rhinoviruses infect human epithelial cells via ceramide-enriched membrane platforms. J Biol Chem. 2005;280(28):26256-26262.

47. Gray LR, Tompkins SC, Taylor EB. Regulation of pyruvate metabolism and human disease. Cell Mol Life Sci. 2014;71(14):2577-2604.

48. Greber BJ, Ban N. Structure and function of the mitochondrial ribosome. Annu Rev Biochem. 2016;85:103-132.

49. Greseth MD, Traktman P. De novo fatty acid biosynthesis contributes significantly to establishment of a bioenergetically favorable environment for vaccinia virus infection. PLoS Pathog. 2014;10(3):e1004021.

50. Gudleski-O'Regan N, Greco TM, Cristea IM, Shenk T. Increased expression of LDL receptor-related protein 1 during human cytomegalovirus infection reduces virion cholesterol and infectivity. Cell Host Microbe. 2012;12(1):86-96.

51. Gullberg RC, Steel JJ, Pujari V, Rovnak J, Crick DC, Perera R. Stearoly-CoA desaturase 1 differentiates early and advanced dengue virus infections and determines virus particle infectivity. PLoS Pathog. 2018;14(8):e1007261.

52. Hackett SR, Zanotelli VR, Xu W, et al. Systems-level analysis of mechanisms regulating yeast metabolic flux. Science. 2016;354(6311):aaf2786.

53. Hafez AY, Messinger JE, McFadden K, et al. Limited nucleotide pools restrict Epstein-Barr virus-mediated B-cell immortalization. Oncogenesis. 2017;6(6):e349.

54. Hammache D, Pieroni G, Yahi N, et al. Specific interaction of HIV-1 and HIV-2 surface envelope glycoproteins with monolayers of galactosylceramide and ganglioside GM3. J Biol Chem. 1998;273(14):7967-7971.

55. Harayama T, Riezman H. Understanding the diversity of membrane lipid composition. Nat Rev Mol Cell Biol. 2018;19(5):281-296.

56. Hatzivassiliou G, Zhao F, Bauer DE, et al. ATP citrate lyase inhibition can suppress tumor cell growth. Cancer Cell. 2005;8(4):311-321.

57. Heaton NS, Perera R, Berger KL, et al. Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases cellular fatty acid synthesis. Proc Natl Acad Sci U S A. 2010;107(40):17345-17350.

58. Heaton NS, Randall G. Dengue virus-induced autophagy regulates lipid metabolism. Cell Host Microbe. 2010;8(5):422-432.

59. Holmes DL, Vogt DT, Lagunoff M. A CRISPR-Cas9 screen identifies mitochondrial translation as an essential process in latent KSHV infection of human endothelial cells. Proc Natl Acad Sci U S A. 2020;117(45):28384-28392.

60. Hopcraft SE, Damania B. Tumour viruses and innate immunity. Philos Trans R Soc Lond B Biol Sci. 2017;372(1732):20160267.

61. Huang H, Li Y, Sadaoka T, et al. Human herpesvirus 6 envelope cholesterol is required for virus entry. J Gen Virol. 2006;87(Pt 2):277-285.

62. Huang S, Czech MP. The GLUT4 glucose transporter. Cell Metab. 2007;5(4):237-252.

63. Huangyang P, Simon MC. Hidden features: exploring the non-canonical functions of metabolic enzymes. Dis Model Mech. 2018;11(8):dmm033365.

64. Hui S, Ghergurovich JM, Morscher RJ, et al. Glucose feeds the TCA cycle via circulating lactate. Nature. 2017;551(7678):115-118.

65. Ilnytska O, Santiana M, Hsu NY, et al. Enteroviruses harness the cellular endocytic machinery to remodel the host cell cholesterol landscape for effective viral replication. Cell Host Microbe. 2013;14(3):281-293.

66. Ioannou MS, Jackson J, Sheu SH, et al. Neuron-astrocyte metabolic coupling protects against activity-induced fatty acid toxicity. Cell. 2019;177(6):1522-1535 e14.

67. Islam MS, Leissing TM, Chowdhury R, Hopkinson RJ, Schofield CJ. 2-oxoglutarate-dependent oxygenases. Annu Rev Biochem. 2018;87:585-620.

68. Isler JA, Skalet AH, Alwine JC. Human cytomegalovirus infection activates and regulates the unfolded protein response. J Virol. 2005;79(11):6890-6899.

69. Jiang H, Badralmaa Y, Yang J, Lempicki R, Hazen A, Natarajan V. Retinoic acid and liver X receptor agonist synergistically inhibit HIV infection in CD4+ T cells by up-regulating ABCA1-mediated cholesterol efflux. Lipids Health Dis. 2012;11:69.

70. Joost HG, Bell GI, Best JD, et al. Nomenclature of the GLUT/SLC2A family of sugar/polyol transport facilitators. Am J Physiol Endocrinol Metab. 2002;282(4):E974-E976.

71. Kleinehr J, Wilden JJ, Boergeling Y, Ludwig S, Hrincius ER. Metabolic modifications by common respiratory viruses and their potential as new antiviral targets. Viruses. 2021;13(10):2068.

72. Krump NA, You J. Molecular mechanisms of viral oncogenesis in humans. Nat Rev Microbiol. 2018;16(11):684-698.

73. Kwon KM, Oh SE, Kim YE, Han TH, Ahn JH. Cooperative inhibition of RIP1-mediated NF-kappaB signaling by cytomegalovirus-encoded deubiquitinase and inactive homolog of cellular ribonucleotide reductase large subunit. PLoS Pathog. 2017;13(6):e1006423.

74. Lange PT, Jondle CN, Darrah EJ, Johnson KE, Tarakanova VL. LXR Alpha restricts gammaherpesvirus reactivation from latently infected peritoneal cells. J Virol. 2019;93(6):e02071-18.

75. Lange PT, Lagunoff M, Tarakanova VL. Chewing the fat: the conserved ability of DNA viruses to hijack cellular lipid metabolism. Viruses. 2019;11(2):119.

76. Lauritzen HP, Schertzer JD. Measuring GLUT4 translocation in mature muscle fibers. Am J Physiol Endocrinol Metab. 2010;299(2):E169-E179.

77. Levy PL, Duponchel S, Eischeid H, et al. Hepatitis C virus infection triggers a tumor-like glutamine metabolism. Hepatology. 2017;65(3):789-803.

78. Li X, Wu K, Zeng S, et al. Viral infection modulates mitochondrial function. Int J Mol Sci. 2021;22(8):4260.

79. Liefhebber JM, Hague CV, Zhang Q, Wakelam MJ, McLauchlan J. Modulation of triglyceride and cholesterol ester synthesis impairs assembly of infectious hepatitis C virus. J Biol Chem. 2014;289(31):21276-21288.

80. Liu PJ, Balfe P, McKeating JA, Schilling M. Oxygen sensing and viral replication: implications for tropism and pathogenesis. Viruses. 2020;12(11):1213.

81. Liu PS, Wang H, Li X, et al. α-ketoglutarate orchestrates macrophage activation through metabolic and epigenetic reprogramming. Nat Immunol. 2017;18(9):985-994.

82. Liu ST, Sharon-Friling R, Ivanova P, et al. Synaptic vesicle-like lipidome of human cytomegalovirus virions reveals a role for SNARE machinery in virion egress. Proc Natl Acad Sci U S A. 2011;108(31):12869-12874.

83. Liu X, Cooper DE, Cluntun AA, et al. Acetate production from glucose and coupling to mitochondrial metabolism in mammals. Cell. 2018;175(2):502-513.e13.

84. Luengo A, Li Z, Gui DY, et al. Increased demand for NAD(+) relative to ATP drives aerobic glycolysis. Mol Cell. 2021;81(4):691-707 e6.

85. Luo Y, Motamedi N, Magaldi TG, Gee GV, Atwood WJ, DiMaio D. Interaction between Simian virus 40 major capsid protein VP1 and cell surface ganglioside GM1 triggers vacuole formation. MBio. 2016;7(2):e00297.

86. Magistretti PJ, Allaman I. Lactate in the brain: from metabolic end-product to signalling molecule. Nat Rev Neurosci. 2018;19(4):235-249.

87. Makowski L, Chaib M, Rathmell JC. Immunometabolism: from basic mechanisms to translation. Immunol Rev. 2020;295(1):5-14.

88. Mandal P, Nagrani LN, Hernandez L, et al. Multiple autonomous cell death suppression strategies ensure cytomegalovirus fitness. Viruses. 2021;13(9):1707.

89. Martin-Acebes MA, Merino-Ramos T, Blazquez AB, et al. The composition of West Nile virus lipid envelope unveils a role of sphingolipid metabolism in flavivirus biogenesis. J Virol. 2014;88(20):12041-12054.

90. Martínez-Reyes I, Chandel NS. Mitochondrial TCA cycle metabolites control physiology and disease. Nat Commun. 2020;11(1):102.

91. Mashimo T, Pichumani K, Vemireddy V, et al. Acetate is a bioenergetic substrate for human glioblastoma and brain metastases. Cell. 2014;159(7):1603-1614.

92. McArdle J, Moorman NJ, Munger J. HCMV targets the metabolic stress response through activation of AMPK whose activity is important for viral replication. PLoS Pathog. 2012;8(1):e1002502.

93. McFadden K, Hafez AY, Kishton R, et al. Metabolic stress is a barrier to Epstein-Barr virus-mediated B-cell immortalization. Proc Natl Acad Sci U S A. 2016;113(6):E782-E790.

94. McKnight SL. The nucleotide sequence and transcript map of the herpes simplex virus thymidine kinase gene. Nucleic Acids Res. 1980;8(24):5949-5964.

95. Meertens L, Carnec X, Lecoin MP, et al. The TIM and TAM families of phosphatidylserine receptors mediate dengue virus entry. Cell Host Microbe. 2012;12(4):544-557.

96. Mews P, Donahue G, Drake AM, Luczak V, Abel T, Berger SL. Acetyl-CoA synthetase regulates histone acetylation and hippocampal memory. Nature. 2017;546(7658): 381-386.

97. Mondal A, Dawson AR, Potts GK, et al. Influenza virus recruits host protein kinase C to control assembly and activity of its replication machinery. Elife. 2017;6:e26910.

98. Montal Emily D, Dewi R, Bhalla K, et al. PEPCK coordinates the regulation of central carbon metabolism to promote cancer cell growth. Mol Cell. 2015;60(4):571-583.

99. Mookerjee SA, Gerencser AA, Nicholls DG, Brand MD. Quantifying intracellular rates of glycolytic and oxidative ATP production and consumption using extracellular flux measurements. J Biol Chem. 2017;292(17):7189-7207.

100. Mora S, Pessin JE. An adipocentric view of signaling and intracellular trafficking. Diabetes Metab Res Rev. 2002;18(5):345-356.

101. Muller C, Hardt M, Schwudke D, Neuman BW, Pleschka S, Ziebuhr J. Inhibition of cytosolic phospholipase A2alpha impairs an early step of coronavirus replication in cell culture. J Virol. 2018;92(4):e01463-17.

102. Munger J, Bajad SU, Coller HA, Shenk T, Rabinowitz JD. Dynamics of the cellular metabolome during human cytomegalovirus infection. PLoS Pathog. 2006;2(12):e132.

103. Munger J, Bennett BD, Parikh A, et al. Systems-level metabolic flux profiling identifies fatty acid synthesis as a target for antiviral therapy. Nat Biotechnol. 2008;26(10):1179-1186.

104. Murphy MP, O'Neill LAJ. Krebs cycle reimagined: the emerging roles of succinate and itaconate as signal transducers. Cell. 2018;174(4):780-784.

105. Mushtaq M, Darekar S, Kashuba E. DNA Tumor viruses and cell metabolism. Oxid Med Cell Longev. 2016;2016:6468342.

106. Naseri-Nosar P, Nogalski MT, Shenk T. The aryl hydrocarbon receptor facilitates the human cytomegalovirus-mediated G1/S block to cell cycle progression. Proc Natl Acad Sci U S A. 2021;118(12):e2026336118.

107. Ng CG, Coppens I, Govindarajan D, Pisciotta J, Shulaev V, Griffin DE. Effect of host cell lipid metabolism on alphavirus replication, virion morphogenesis, and infectivity. Proc Natl Acad Sci U S A. 2008;105(42):16326-16331.

108. Noch E, Khalili K. Oncogenic viruses and tumor glucose metabolism: like kids in a candy store. Mol Cancer Ther. 2012;11(1):14-23.

109. Nussinov R, Tsai CJ, Jang H. Dynamic protein allosteric regulation and disease. Adv Exp Med Biol. 2019;1163:25-43.

110. Otto JC, Casey PJ. The hepatitis delta virus large antigen is farnesylated both in vitro and in animal cells. J Biol Chem. 1996;271(9):4569-4572.

111. Park JO, Rubin SA, Xu YF, et al. Metabolite concentrations, fluxes and free energies imply efficient enzyme usage. Nat Chem Biol. 2016;12(7):482-489.

112. Passalacqua KD, Lu J, Goodfellow I, et al. Glycolysis is an intrinsic factor for optimal replication of a norovirus. MBio. 2019;10(2):e02175-18.

113. Patrone M, Percivalle E, Secchi M, et al. The human cytomegalovirus UL45 gene product is a late, virion-associated protein and influences virus growth at low multiplicities of infection. J Gen Virol. 2003;84(Pt 12):3359-3370.

114. Pavlova NN, Thompson CB. The emerging hallmarks of cancer metabolism. Cell Metab. 2016;23(1):27-47.

115. Preston VG, Fisher FB. Identification of the herpes simplex virus type 1 gene encoding the dUTPase. Virology. 1984;138(1):58-68.

116. Preston VG, Palfreyman JW, Dutia BM. Identification of a herpes simplex virus type 1 polypeptide which is a component of the virus-induced ribonucleotide reductase. J Gen Virol. 1984;65(Pt 9):1457-1466.

117. Prusinkiewicz MA, Mymryk JS. Metabolic reprogramming of the host cell by human adenovirus infection. Viruses. 2019;11(2):141.

118. Purdy JG, Luftig MA. Reprogramming of cellular metabolic pathways by human oncogenic viruses. Curr Opin Virol. 2019;39:60-69.

119. Purdy JG, Shenk T, Rabinowitz JD. Fatty acid elongase 7 catalyzes lipidome remodeling essential for human cytomegalovirus replication. Cell Rep. 2015;10(8):1375-1385.

120. Pyles RB, Sawtell NM, Thompson RL. Herpes simplex virus type 1 dUTPase mutants are attenuated for neurovirulence, neuroinvasiveness, and reactivation from latency. J Virol. 1992;66(11):6706-6713.

121. Rajasagi NK, Reddy PB, Suryawanshi A, Mulik S, Gjorstrup P, Rouse BT. Controlling herpes simplex virus-induced ocular inflammatory lesions with the lipid-derived mediator resolvin E1. J Immunol. 2011;186(3):1735-1746.

122. Ranneberg-Nilsen T, Dale HA, Luna L, et al. Characterization of human cytomegalovirus uracil DNA glycosylase (UL114) and its interaction with polymerase processivity factor (UL44). J Mol Biol. 2008;381(2):276-288.

123. Read SA, Tay E, Shahidi M, George J, Douglas MW. Hepatitis C virus infection mediates cholesteryl ester synthesis to facilitate infectious particle production. J Gen Virol. 2014;95(Pt 9):1900-1910.

124. Reeves MB, Davies AA, McSharry BP, Wilkinson GW, Sinclair JH. Complex I binding by a virally encoded RNA regulates mitochondria-induced cell death. Science. 2007;316(5829):1345-1348.

125. Ripperger TJ, Bhattacharya D. Transcriptional and metabolic control of memory B cells and plasma cells. Annu Rev Immunol 2021;39:345-368.

126. Rousso I, Mixon MB, Chen BK, Kim PS. Palmitoylation of the HIV-1 envelope glycoprotein is critical for viral infectivity. Proc Natl Acad Sci U S A. 2000;97(25):13523-13525.

127. Sanchez EL, Carroll PA, Thalhofer AB, Lagunoff M. Latent KSHV infected endothelial cells are glutamine addicted and require glutaminolysis for survival. PLoS Pathog. 2015;11(7):e1005052.

128. Sanchez EL, Lagunoff M. Viral activation of cellular metabolism. Virology. 2015;479-480:609-618.

129. Sanchez EL, Pulliam TH, Dimaio TA, Thalhofer AB, Delgado T, Lagunoff M. Glycolysis, glutaminolysis, and fatty acid synthesis are required for distinct stages of Kaposi's sarcoma-associated herpesvirus lytic replication. J Virol. 2017;91(10):e02237-16.

130. Sanchez V, Dong JJ. Alteration of lipid metabolism in cells infected with human cytomegalovirus. Virology. 2010;404(1):71-77.

131. Scagliola A, Mainini F, Cardaci S. The tricarboxylic acid cycle at the crossroad between cancer and immunity. Antioxid Redox Signal. 2020;32(12):834-852.

132. Schug ZT, Peck B, Jones DT, et al. Acetyl-CoA synthetase 2 promotes acetate utilization and maintains cancer cell growth under metabolic stress. Cancer Cell. 2015;27(1): 57-71.

133. Selak MA, Armour SM, MacKenzie ED, et al. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. Cancer Cell. 2005;7(1):77-85.

134. Sheng XX, Sun YJ, Zhan Y, et al. The LXR ligand GW3965 inhibits Newcastle disease virus infection by affecting cholesterol homeostasis. Arch Virol. 2016;161(9):2491-2501.

135. Shenk T, Alwine JC. Human cytomegalovirus: coordinating cellular stress, signaling, and metabolic pathways. Annu Rev Virol. 2014;1(1):355-374.

136. Slatter DA, Aldrovandi M, O'Connor A, et al. Mapping the human platelet lipidome reveals cytosolic phospholipase A2 as a regulator of mitochondrial bioenergetics during activation. Cell Metab. 2016;23(5):930-944.

137. Smallwood HS, Duan S, Morfouace M, et al. Targeting metabolic reprogramming by influenza infection for therapeutic intervention. Cell Rep. 2017;19(8):1640-1653.

138. Smidansky ED, Arnold JJ, Reynolds SL, Cameron CE. Human mitochondrial RNA polymerase: evaluation of the single-nucleotide-addition cycle on synthetic RNA/DNA scaffolds. Biochemistry. 2011;50(22):5016-5032.

139. Smith JS, Lefkowitz RJ, Rajagopal S. Biased signalling: from simple switches to allosteric microprocessors. Nat Rev Drug Discov. 2018;17(4):243-260.

140. Smith M, Honce R, Schultz-Cherry S. Metabolic syndrome and viral pathogenesis: lessons from influenza and coronaviruses. J Virol. 2020;94(18):e00665-20.

141. Solano Fonseca R, Metang P, Egge N, et al. Glycolytic preconditioning in astrocytes mitigates trauma-induced neurodegeneration. Elife. 2021;10:e69438.

142. Sousa IP Jr, Carvalho CA, Ferreira DF, et al. Envelope lipid-packing as a critical factor for the biological activity and stability of alphavirus particles isolated from mammalian and mosquito cells. J Biol Chem. 2011;286(3):1730-1736.

143. Spencer CM, Schafer XL, Moorman NJ, Munger J. Human cytomegalovirus induces the activity and expression of acetyl-coenzyme A carboxylase, a fatty acid biosynthetic enzyme whose inhibition attenuates viral replication. J Virol. 2011;85(12):5814-5824.

144. Stern-Ginossar N, Thompson SR, Mathews MB, Mohr I. Translational control in virus-infected cells. Cold Spring Harb Perspect Biol. 2019;11(3):a033001.

145. Stoeck IK, Lee JY, Tabata K, et al. Hepatitis C virus replication depends on endosomal cholesterol homeostasis. J Virol. 2018;92(1):e01196-17.

146. Sun X, Whittaker GR. Role for influenza virus envelope cholesterol in virus entry and infection. J Virol. 2003;77(23):12543-12551.

147. Sutendra G, Kinnaird A, Dromparis P, et al. A nuclear pyruvate dehydrogenase complex is important for the generation of acetyl-CoA and histone acetylation. Cell. 2014;158(1):84-97.

148. Sychev ZE, Hu A, DiMaio TA, et al. Integrated systems biology analysis of KSHV latent infection reveals viral induction and reliance on peroxisome mediated lipid metabolism. PLoS Pathog. 2017;13(3):e1006256.

149. Tang C, Loeliger E, Luncsford P, Kinde I, Beckett D, Summers MF. Entropic switch regulates myristate exposure in the HIV-1 matrix protein. Proc Natl Acad Sci U S A. 2004;101(2):517-522.

150. Tannahill GM, Curtis AM, Adamik J, et al. Succinate is an inflammatory signal that induces IL-1β through HIF-1α. Nature. 2013;496(7444):238-242.

151. Tanner LB, Chng C, Guan XL, Lei Z, Rozen SG, Wenk MR. Lipidomics identifies a requirement for peroxisomal function during influenza virus replication. J Lipid Res. 2014;55(7):1357-1365.

152. Terry LJ, Vastag L, Rabinowitz JD, Shenk T. Human kinome profiling identifies a requirement for AMP-activated protein kinase during human cytomegalovirus infection. Proc Natl Acad Sci U S A. 2012;109(8):3071-3076.

153. TeSlaa T, Bartman CR, Jankowski CSR, et al. The source of glycolytic intermediates in mammalian tissues. Cell Metab. 2021;33(2):367-378 e5.

154. Thaker SK, Ch'ng J, Christofk HR. Viral hijacking of cellular metabolism. BMC Biol. 2019;17(1):59.

155. Thompson CB. Rethinking the regulation of cellular metabolism. Cold Spring Harb Symp Quant Biol. 2011;76:23-29.

156. Thompson CB, Bielska AA. Growth factors stimulate anabolic metabolism by directing nutrient uptake. J Biol Chem. 2019;294(47):17883-17888.

157. Tiku V, Tan MW, Dikic I. Mitochondrial functions in infection and immunity. Trends Cell Biol. 2020;30(4):263-275.

158. Tsai K, Messick TE, Lieberman PM. Disruption of host antiviral resistances by gammaherpesvirus tegument proteins with homology to the FGARAT purine biosynthesis enzyme. Curr Opin Virol. 2015;14:30-40.

159. Turnell AS, Grand RJ. DNA viruses and the cellular DNA-damage response. J Gen Virol. 2012;93(Pt 10):2076-2097.

160. Uldry M, Thorens B. The SLC2 family of facilitated hexose and polyol transporters. Pflugers Arch. 2004;447(5):480-489.

161. Van Skike ND, Minkah NK, Hogan CH, et al. Viral FGARAT ORF75A promotes early events in lytic infection and gammaherpesvirus pathogenesis in mice. PLoS Pathog. 2018;14(2):e1006843.

162. van Teijlingen Bakker N, Pearce EJ. Cell-intrinsic metabolic regulation of mononuclear phagocyte activation: findings from the tip of the iceberg. Immunol Rev. 2020;295(1):54-67.

163. Vastag L, Koyuncu E, Grady SL, Shenk TE, Rabinowitz JD. Divergent effects of human cytomegalovirus and herpes simplex virus-1 on cellular metabolism. PLoS Pathog. 2011;7(7):e1002124.

164. Vienberg S, Geiger J, Madsen S, Dalgaard LT. MicroRNAs in metabolism. Acta Physiol (Oxf). 2017;219(2):346-361.

165. Vijay R, Hua X, Meyerholz DK, et al. Critical role of phospholipase A2 group IID in age-related susceptibility to severe acute respiratory syndrome-CoV infection. J Exp Med. 2015;212(11):1851-1868.

166. Vijayan M, Seo YJ, Pritzl CJ, Squires SA, Alexander S, Hahm B. Sphingosine kinase 1 regulates measles virus replication. Virology. 2014;450-451:55-63.

167. Vysochan A, Sengupta A, Weljie AM, Alwine JC, Yu Y. ACSS2-mediated acetyl-CoA synthesis from acetate is necessary for human cytomegalovirus infection. Proc Natl Acad Sci U S A. 2017;114(8):E1528-E1535.

168. Wallace M, Green CR, Roberts LS, et al. Enzyme promiscuity drives branched-chain fatty acid synthesis in adipose tissues. Nat Chem Biol. 2018;14(11):1021-1031.

169. Wang C, Gale M Jr, Keller BC, et al. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. Mol Cell. 2005;18(4):425-434.

170. Wang LW, Shen H, Nobre L, et al. Epstein-Barr-virus-induced one-carbon metabolism drives B cell transformation. Cell Metab. 2019;30(3):539-555 e11.

171. Wang LW, Wang Z, Ersing I, et al. Epstein-Barr virus subverts mevalonate and fatty acid pathways to promote infected B-cell proliferation and survival. PLoS Pathog. 2019;15(9):e1008030.

172. Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. J Gen Physiol. 1927;8(6):519-530.

173. Ward PS, Patel J, Wise DR, et al. The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. Cancer Cell. 2010;17(3):225-234.

174. Watanabe H, Takehana K, Date M, Shinozaki T, Raz A. Tumor cell autocrine motility factor is the neuroleukin/phosphohexose isomerase polypeptide. Cancer Res. 1996;56(13):2960-2963.

175. Welsch F, Wenger WC, Stedman DB. Choline metabolism in placenta: evidence for the biosynthesis of phosphatidylcholine in microsomes via the methylation pathway. Placenta. 1981;2(3):211-221.

176. Wichit S, Hamel R, Bernard E, et al. Imipramine inhibits chikungunya virus replication in human skin fibroblasts through interference with intracellular cholesterol trafficking. Sci Rep. 2017;7(1):3145.

177. Williams AN, Sherman MB, Smith HQ, et al. A norovirus uses bile salts to escape antibody recognition while enhancing receptor binding. J Virol. 2021;95(13):e0017621.

178. Wise LM, Xi Y, Purdy JG. Hypoxia-Inducible Factor 1α(HIF1α) suppresses virus replication in human cytomegalovirus infection by limiting kynurenine synthesis. MBio. 2021;12(2):e02956-20.

179. Wu YH, Yang Y, Chen CH, et al. Aerobic glycolysis supports hepatitis B virus protein synthesis through interaction between viral surface antigen and pyruvate kinase isoform M2. PLoS Pathog. 2021;17(3):e1008866.

180. Wudiri GA, Nicola AV. Cellular cholesterol facilitates the postentry replication cycle of herpes simplex virus 1. J Virol. 2017;91(14):e00445-17.

181. Xi Y, Harwood S, Wise L, Purdy JG. Human cytomegalovirus pUL37x1 is important to remodeling of host lipid metabolism. J Virol. 2019;93(21):1-19.

182. Xi Y, Lindenmayer L, Kline I, von Einem J, Purdy JG. Human cytomegalovirus uses a host stress response to balance the elongation of saturated/monounsaturated and polyunsaturated very-long-chain fatty acids. MBio. 2021;12(3):e00167-21.

183. Xu K, Nagy PD. RNA virus replication depends on enrichment of phosphatidylethanolamine at replication sites in subcellular membranes. Proc Natl Acad Sci U S A. 2015;112(14):E1782-E1791.

184. Xu K, Nagy PD. Enrichment of phosphatidylethanolamine in viral replication compartments via co-opting the endosomal Rab5 small GTPase by a positive-strand RNA virus. PLoS Biol. 2016;14(10):e2000128.

185. Yan B, Chu H, Yang D, et al. Characterization of the lipidomic profile of human coronavirus-infected cells: implications for lipid metabolism remodeling upon coronavirus replication. Viruses. 2019;11(1):73.

186. Ying M, You D, Zhu X, Cai L, Zeng S, Hu X. Lactate and glutamine support NADPH generation in cancer cells under glucose deprived conditions. Redox Biol. 2021;46:102065.

187. Yore MM, Syed I, Moraes-Vieira PM, et al. Discovery of a class of endogenous mammalian lipids with anti-diabetic and anti-inflammatory effects. Cell. 2014;159(2):318-332.

188. Yu L, Chen X, Wang L, Chen S. Oncogenic virus-induced aerobic glycolysis and tumorigenesis. J Cancer. 2018;9(20):3699-3706.

189. Yu Y, Maguire TG, Alwine JC. Human cytomegalovirus activates glucose transporter 4 expression to increase glucose uptake during infection. J Virol. 2011;85(4):1573-1580.

190. Yu Y, Maguire TG, Alwine JC. Human cytomegalovirus infection induces adipocyte-like lipogenesis through activation of sterol regulatory element binding protein 1. J Virol. 2012;86(6):2942-2949.

191. Yu Y, Maguire TG, Alwine JC. ChREBP, a glucose-responsive transcriptional factor, enhances glucose metabolism to support biosynthesis in human cytomegalovirus-infected cells. Proc Natl Acad Sci U S A. 2014;111(5):1951-1956.

192. Yu Y, Pierciey FJ Jr, Maguire TG, Alwine JC. PKR-like endoplasmic reticulum kinase is necessary for lipogenic activation during HCMV infection. PLoS Pathog. 2013;9(4):e1003266.

193. Zhang J, Zhang Z, Chukkapalli V, et al. Positive-strand RNA viruses stimulate host phosphatidylcholine synthesis at viral replication sites. Proc Natl Acad Sci U S A. 2016;113(8):E1064-E1073.

194. Zhu H, Cong JP, Yu D, Bresnahan WA, Shenk TE. Inhibition of cyclooxygenase 2 blocks human cytomegalovirus replication. Proc Natl Acad Sci U S A. 2002;99(6):3932-3937.

195. Zhu J, Thompson CB. Metabolic regulation of cell growth and proliferation. Nat Rev Mol Cell Biol. 2019;20(7):436-450.

CHAPTER 8

Pathogenesis of Viral Infection

Thomas E. Morrison • Mark T. Heise

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Introduction to Viral Pathogenesis

The term viral pathogenesis generally describes the processes by which a virus causes disease in a host. Different viruses cause distinct disease manifestations, due to factors such as virus-specific differences in the cell type or tissue targets of replication, the viral replication cycle, viral effects on the infected cell, and the host response to viral infection. Furthermore, infection with the same virus can result in outcomes ranging from asymptomatic infection to lethal disease across different individuals, further blurring the border between infection and disease. These differences in disease outcome are the result of many complex interacting factors that are unique to each specific virus, a particular host species, and the individual host. The interplay of these factors determines whether infection is successful, whether disease occurs, and if so, the severity and duration of disease.

The past two decades have seen a resurgence in interest in viral pathogenesis driven in large part by the emergence of several significant viral disease threats, including Ebola virus, Zika virus (ZIKV), and most notably, SARS-CoV-2. These outbreaks have led to the recognition that the development of safe and effective treatments and vaccines against these pathogens requires an understanding of their pathogenesis. The renewed interest in viral pathogenesis coincided with a molecular biology revolution that has seen the development and rapid deployment of new methods for quantifying viral and host gene expression (eg, advanced proteomics, RNAseq, spatial transcriptomics), high throughput methods for manipulating host gene expression (eg, CRISPR), as well as tools for rapidly engineering viral genomes through the application of synthetic biology and viral molecular clone technologies that have allowed the molecular manipulation of most clinically relevant virus families. These methods have provided the virology field with an unprecedented ability to manipulate both viral and host genomes and study the molecular events involved in every stage of the virus-induced disease process. These include the identification of entry receptors, pro and antiviral host factors, and polymorphic host genes that govern cell tropism, host range, and disease severity for a number of important viral pathogens, while also identifying key viral genetic determinants that interact with these host factors and regulate viral virulence. Furthermore, *ex vivo* systems for growing and manipulating primary host cells or even organ systems have led to significant advances in our understanding of how viruses interact with target cells. However, while these reductionist tools and systems have been invaluable for understanding the role that specific viral and host factors play in the virus-induced disease process, viral pathogenesis involves a complex set of interactions between viral and host factors, making it necessary to validate the results of these *ex vivo* studies in whole organisms.

Many aspects of virus-induced disease, including interactions with the host immune system, involve systemic responses and can therefore only be fully studied in the whole organism. Ideally, these responses should be studied in the natural host, and studies in virally infected natural hosts, including humans, have provided significant insights into the pathogenesis of a wide variety of clinically relevant viruses. However, for human viruses, it is often not possible to perform the types of highly invasive assays needed to mechanistically understand virus-induced disease processes within the infected individual. It is also difficult to control for the wide array of factors, such as viral dose, preexisting immunity, or other environmental factors that can significantly impact disease outcome during natural infection. Therefore, viral pathogenesis research is often conducted in model organisms of virus-induced disease. These model systems allow for better control of viral (eg, viral strain, dose) and host (eg, genetics, infection history, microbiome) factors that can confound analysis of natural infection, while permitting more mechanistic analysis through manipulation of the virus or the host. These model systems have proven to be invaluable for studying the pathogenesis of a wide range of viruses and have provided insights into how viral interactions with the host affect every aspect of the virus-induced disease process. There is also a growing recognition of the limitations of existing model systems and both the need for improved models that better reflect infection in the natural host and the necessity of confirming results in the natural host (eg, humans). Given the importance of understanding the pathogenesis of emerging viruses, and the rapid advances in molecular virology and animal models of disease, the study of viral pathogenesis continues to be an important and rapidly moving research area.

Goals of This Chapter

The focus of this chapter will be to utilize classical examples of viral pathogenesis, while discussing new insights into the molecular biology and molecular pathogenesis of disease-associated viruses to illustrate key concepts in viral pathogenesis. The large number of viral emergence events that have occurred over that past 40 years, first exemplified by human immunodeficiency virus (HIV), and including more recent outbreaks of ZIKV and Ebola virus, as well as the emergence of SARS-CoV-2 in 2019, have driven a significant expansion in our knowledge of how viruses cause disease. However, it is important to note that viral pathogenesis has been studied for well over 100 years, and this work has been covered in a variety of other authoritative texts that summarize many of the initial observations that form the foundation of our understanding of viral pathogenesis.[237,342] Not only do these early studies provide important context for understanding the evolution of the viral pathogenesis field, but they illustrate the importance of understanding viral pathogenesis for the development of effective public health interventions, including vaccines against human pathogens such as poliovirus, measles virus, hepatitis B virus (HBV), and SARS-CoV-2 or antiviral drugs against viruses such as HIV, hepatitis C virus (HCV), and SARS-CoV-2.

Because viral pathogenesis encompasses a wide variety of viruses and virus-induced disease states, it is not possible to provide a detailed description of the pathogenesis of all viral infections in a single chapter. Therefore, the goal of this chapter is to provide a conceptual framework for understanding viral pathogenesis. To achieve this goal, we will define common terms used in the study of viral pathogenesis, discuss research areas/tools for studying viral pathogenesis, and present key concepts using examples from a range of viral infections. Importantly, for details on the pathogenesis of specific viruses, the reader should refer to chapters in this book series that are devoted to these specific viruses. Furthermore, while viral infections impact a broad range of nonmammalian species, and much of our understanding of virus/host interactions has come from studies of viruses that affect plants or other nonmammalian species, this chapter will specifically focus on viruses affecting mammals, with an emphasis on viruses that are associated with human disease, or viruses that have served as robust model systems for human disease, or as models for studying foundational principals of viral pathogenesis.

Definitions and Concepts in Viral Pathogenesis

As noted above, the field of viral pathogenesis is over 100 years old and is associated with an extremely large body of literature. Due to the breadth of the field, many of the key terms used to discuss topics in viral pathogenesis have multiple definitions across the viral pathogenesis literature. Therefore, we will start by defining key terms and concepts as they are used in this chapter.

Productive, Abortive, and Latent Infection

Infection describes the processes whereby a virus introduces its genome into a host cell. Infection only occurs if the virus is able to bind to and introduce its genetic material into the cell. Infection is ***productive*** if the virus is able to complete its replication cycle to produce new virus, while no progeny virus is produced during ***abortive*** infection. A cell is described as ***susceptible*** if it has a functional receptor or receptors (multiple may be required) that supports viral entry and ***permissive*** if it is capable of supporting productive infection, and nonpermissive if infection is abortive. A susceptible cell may be permissive or nonpermissive. An infection is defined as ***latent*** if the virus infects a cell and does not immediately produce progeny virus but maintains the ability to produce progeny virus at a later time via a process termed reactivation. Importantly, latency is a unique stage in the virus life cycle with a distinct pattern of viral gene expression that differentiates it from productive infection. Productive infection can be ***cytolytic*** (cytocidal), resulting in death of the host cell, while ***noncytolytic*** infections produce progeny viruses without directly killing the cell. Individual viruses can produce cytolytic and noncytolytic infections depending on the cell type and context (eg, *in vitro* vs *in vivo*).

Acute vs Chronic Infection

Acute infection occurs when a virus first infects a susceptible host (Fig. 8.1), while ***chronic infection*** is defined as ongoing infection beyond the period when the host immune system would be expected to clear acute infection. Chronic infection is also referred to as "persistent" infection, and the terms can be used interchangeably. Chronic infection can involve continuous viral replication or latency/reactivation. During latent viral infection the virus has a distinct gene expression strategy, often involving restricted viral gene expression, which allows the genome to survive even when lytic replication is not occurring. Examples of chronic infections include the proviral form of retroviruses, or the circular episomal form with selective expression of viral genes observed for herpesviruses such as Epstein-Barr virus (EBV) and herpes simplex virus (HSV). Often latently infected cells express no viral proteins, making latency immunologically silent. For viruses like HIV, which persists in both a latent form while maintaining continuous replication,[187,510] the boundary between acute and chronic infection is defined as the period when the host immune response clears the initial burst of infection and a relatively stable viral load set point is established in the infected host. For some viruses, such as herpesviruses, infection of a permissive host almost always results in latency. However, there is significant variation in the development of persistent viral infection with other viruses. For example, while HBV frequently causes persistent infection, only a subset of individuals are chronically infected while others clear the infection.[211] Viral persistence can also occur for prolonged periods of time without the production of detectable infectious virus or reactivation competent virus. While the mechanisms underlying persistence of some RNA viruses are not completely understood, potential mechanisms include abortive infection, generation of defective viruses, or low levels of ongoing replication that fall below the sensitivity of standard detection methods. While these types of persistent infection do not result in the generation of infectious virus, they can be associated with significant pathology, such as subacute sclerosing panencephalitis (SSPE), a progressively lethal disease that is caused by hypermutated defective measles virus in the central nervous system (CNS).[178]

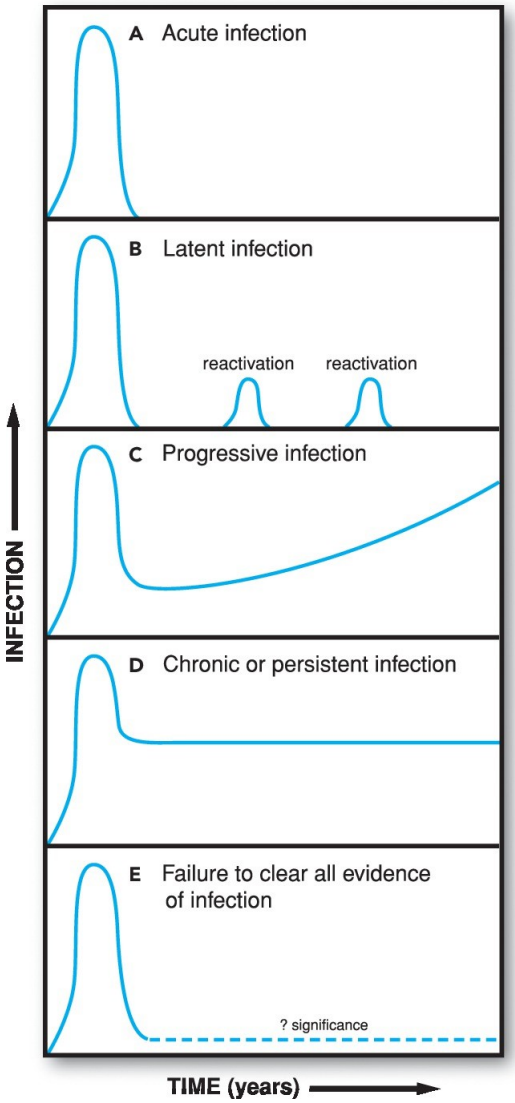

FIGURE 8.1 **Schematic of different patterns of viral infection**. **A**. Acute infection occurs when a virus first infects a susceptible host. All evidence of infection is cleared. **B**. Latent infection, characterized by intermittent viral reactivation, is a mechanism of chronic infection. **C**. Progressive infection is a form of chronic infection in which the viral burden continuously expands over time. **D**. Chronic or persistent infection is the continuation of infection beyond the time when the immune system is expected to clear acute infection. **E**. In some cases, viral nucleic acid can be detected in the host for prolonged time periods but the nature of the infectious process has not been defined. (This figure was adapted from Chapter 22 in Fields Virology, Third Edition by Herbert W. Virgin IV.)

Quasispecies

Viral variants arise due to errors that occur during viral RNA or DNA synthesis, and the population of variants constituting the virus population is referred to as a viral quasispecies (Fig. 8.2). While both DNA and RNA viruses exist as quasispecies, error-prone RNA dependent RNA polymerases (RdRp) can result in the rapid generation of genetic variants in an RNA virus population, and there is generally more genetic diversity in RNA viruses. Studies with poliovirus demonstrated that viruses with enhanced RdRP fidelity that limited the generation of viral mutants showed decreased virulence,[505] and viral variants in a population may exhibit altered cell or tissue tropism, affect host range through interactions with cellular receptors or other proviral factors, and facilitate escape from immune pressure. Therefore, while many areas within the virology and viral pathogenesis fields have focused on studying clonal, infectious clone derived viruses, it is important to consider the potential impact of quasispecies on viral fitness, host range, and pathogenesis.

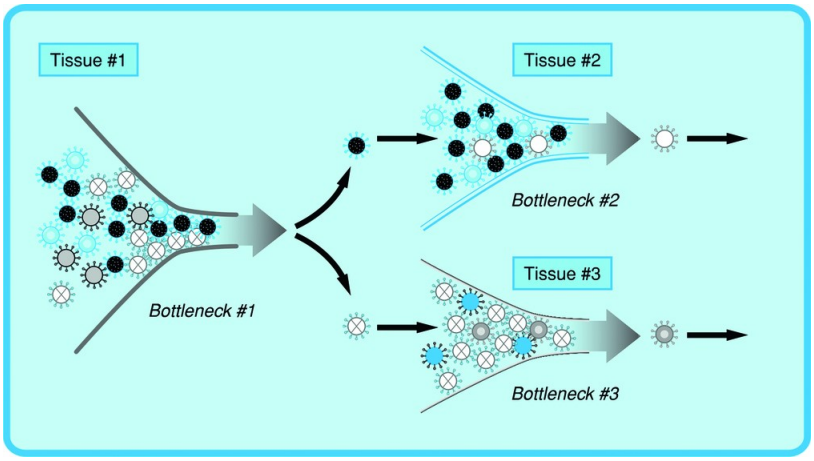

FIGURE 8.2 **Viral pathogenesis as stochastic events followed by selection by bottlenecks in infection**. Shown is a representation of a viral quasispecies, only two of which are fit to pass through an initial bottleneck in infection. Subsequent replication of these viruses in different tissues generates further quasispecies that are again acted on by selective forces at further bottlenecks in infection. (This figure was adapted from Chapter 22 in Fields Virology, Third Edition by Herbert W. Virgin IV.)

Host Response to Acute vs Chronic Infection

Some viruses (eg, herpesviruses) cause both acute and persistent infection in the same individual, while other viruses (eg, HBV, human papillomavirus [HPV]) may be cleared following acute infection in some individuals but progress to chronic infection in others. The host immune response plays a major role in controlling and clearing acute infection and maintains equilibrium during chronic infection. Therefore, knowledge of how the host immune response controls acute infections and regulates chronic infections is essential for understanding the pathogenesis of virus-induced disease. For many viruses, the immune effectors that mediate acute viral clearance are different than those that regulate chronic infection/latency. For example, interferon gamma is an essential regulator of murine gamma herpesvirus 68 (γHV68) latency, but is nonessential for control of acute infection.[33] These differences in immune control between acute and chronic infection may reflect the differential viral gene expression programs between acute replication and latency that characterize herpesvirus infections. However, for viruses that replicate continuously, maintenance of chronic infection generally involves evasion of host immune responses through other mechanisms. For example, both HIV and HCV generate large numbers of viral variants that evade the host immune response and prevent viral clearance.[70,223] Chronic infections with viruses, such as lymphocytic choriomeningitis virus (LCMV), HIV, and HCV, may overwhelm the capacity of the host immune system to clear the virus, leading to immune exhaustion and viral persistence.[229] Therefore, chronic infection should not be viewed as a simple extension of acute infection, since the two stages often involve very different interactions between the host immune system and the virus.

Disease

Virus-Induced Disease is defined as a harmful pathologic consequence of infection. Since viral pathogenesis is the study of virus-induced disease, for the purposes of this chapter we are only concerned with viruses where infection has the capacity to cause disease in at least a subset of infected individuals. With a few notable exceptions (eg, rabies virus), infection with most disease-associated viruses results in a range of outcomes spanning asymptomatic infection to severe disease or death. Some viruses, such as rabies virus, almost always result in lethal disease. However, even viruses such as Ebola virus that were thought to cause predominantly severe disease, similar to rabies virus, actually cause a spectrum of disease manifestations ranging from mild disease to hemorrhagic fever or multi-organ failure.[219] Often, the full spectrum of disease manifestations elicited by a particular virus is only revealed by outbreaks of substantial magnitude, such as the 2014-2016 Ebola virus outbreak in West Africa, the 2015-2017 outbreak of ZIKV in the Americas, and the 2019-present SARS-CoV-2 pandemic. Understanding the factors that drive these differential disease outcomes is a central theme of viral pathogenesis research.

Viruses can cause disease through several distinct mechanisms including direct virus-induced damage, virus-induced immune pathology, paracrine effects of viral infection, and virus-induced cellular dysfunction. Cytolytic viruses cause direct cell death, which can result in severe disease when the target cells perform an essential function and/or are nonrenewable, such as neuron destruction by rabies virus or Eastern equine encephalitis virus (EEEV). While the host immune response is essential for the control and clearance of most viruses, immune pathology due to overactive host immune responses can also cause virus-induced disease, as seen with HBV-induced acute hepatitis, which is largely mediated by the host immune response killing HBV infected hepatocytes.[141,482] Virus-induced disease can also be driven by viral proteins that alter the host cell, such as HPV driven tumor formation,[313] or through paracrine effects of secreted viral proteins, as illustrated by angiogenesis driven by secreted Kaposi sarcoma–associated herpesvirus (KSHV) derived proteins.[534] While most virus-induced disease states involve a combination of several or even all of these processes, understanding the major drivers of disease provides important insights into the biology of the specific virus, while also informing potential therapeutic strategies for that viral infection. For example, chikungunya virus (CHIKV) is

directly cytopathic for infected cells. However, overactive host inflammatory responses mediated by inflammatory monocytes and CD4 T cells contribute to CHIKV-induced arthritis,[477] and combining therapies that block viral replication while also inhibiting host inflammatory response shows promise for treating virus-induced disease.[324]

Virulence

Virulence, which is defined as the capacity of a virus to cause disease, determines the relationship between infection and disease. Avirulent or attenuated viruses are generally associated with mild or asymptomatic disease, while virulent viruses are associated with more frequent and more severe disease. Virulence can take on many forms that are dependent upon the virus-induced disease process. For example, virulent strains of variola virus, the causative agent of smallpox, resulted in rapid death with high case mortality rates, while certain highly virulent HPV strains are associated with high rates of tumor induction. Therefore, it is difficult to compare the virulence of two entirely different viruses (eg, influenza A virus and human cytomegalovirus [HCMV]), and comparisons of viral virulence should be restricted to related viruses. For example, the attenuated Reston strain of Ebola virus appears to be nonvirulent in humans, while Ebola Zaire is highly virulent, with high case mortality rates in infected humans.[538]

While virulence can often be linked to a virus's replication capacity, there are examples where viruses with reduced replication capacity show enhanced virulence[194] and multiple factors contribute to virulence, including the virus's cell and tissue tropism, the virus's ability to antagonize or evade the host innate immune response, and the virus's ability to replicate efficiently. Importantly, the host also plays a major role in determining whether a given virus is virulent, since normally avirulent viruses or even live attenuated vaccine strains can cause severe disease in individuals with highly compromised immune systems.

Although viral virulence genes are associated with a virus's capacity to cause disease, there are also examples of viral genes that actually dampen virus-induced disease. For instance, the vaccinia virus B15R protein is a secreted IL-1β receptor homolog that neutralizes IL-1β. Viruses carrying a mutated form of this gene induced fever and caused increased weight loss and disease signs compared with wild type virus.[8,9] This effect was due to B15R's effects on IL-1β, since administration of anti-IL-1β antibodies to mice infected with the mutant virus reversed the effects of the B15R mutation on fever.[8,9] Therefore, in certain situations, virally encoded genes may limit virus-induced pathogenesis.

Invasiveness

Invasiveness is the capacity of a virus to enter into and damage a tissue, a property that distinguishing viruses with high potential virulence but which differ in the efficiency with which they enter target tissues. For example, many strains of Sindbis virus cause severe neurologic disease in experimental animals if directly inoculated into the CNS, but are avirulent when inoculated into the periphery, while related neuroinvasive viruses with adaptive mutations that allow passage through the blood-brain barrier into the CNS can cause lethal disease following either peripheral or intracranial inoculation.[121]

Proviral and Antiviral Host Factors

Since all viruses are obligate intracellular parasites, they are by definition dependent upon the host cell for the production of all of the components comprising the virion (eg, nucleic acid, proteins). Cellular factors that are essential for or enhance viral infection, replication, or virion production are referred to as ***proviral factors***. Many of these factors are essential for all viruses (eg, ribosomes), while other factors, such as viral entry receptors or cellular cofactors that are co-opted by viruses to promote viral replication may be specific to virus families or even individual viruses. For example, the liver-specific host microRNA miR-122 is essential for robust HCV replication and inhibition of miR-122 in nonhuman primates limits HCV replication.[227,271] While the role of proviral factors has been recognized for decades, the development of high-throughput, whole genome methods for interrogating the importance for individual host genes, such as siRNA gene knockdown or CRISPR gene knockout screens, as well as more sensitive biochemical methods for identifying host proteins that interact with viral proteins, has led to significant advances in the identification of proviral host factors that facilitate viral replication, including new entry receptors, enzymatic pathways that are essential for viral growth, and cellular proteins that interact with viral replication complexes or facilitate the generation of viral replication compartments.[272,302,544] Not only has this knowledge led to new insights into how specific host factors regulate viral cell tropism or even host range, this information has also reinforced the idea that inhibiting essential cellular factors may have therapeutic benefit for treating virus-induced disease.[265]

While proviral factors are essential for successful viral infection, other cellular factors actively antagonize viral replication to prevent or limit infection. These ***antiviral factors*** include constitutively expressed restriction factors that can limit the ability of specific viruses to replicate efficiently in a species-specific manner, as well as inducible antiviral factors, such as interferon stimulated genes (ISGs). While some restriction factors show activity against a limited number of viruses, many antiviral genes have an essential protective role against a wide range of viruses.[233,234,266,432,433] In some cases, specific genes can determine the host range of highly pathogenic viruses such as ZIKV, yellow fever virus (YFV), or HIV.[168,306] Furthermore, naturally occurring variation in specific antiviral genes or their regulatory pathways can have a major impact on an individual's susceptibility to specific viruses.[91,92,288,409,542]

Viral Immune Evasion

Most viruses employ mechanisms to either actively antagonize or avoid detection by the host innate and adaptive immune systems or to bypass intrinsic cellular resistance molecules (also known as restriction factors) so that the virus can complete the infectious process and spread to a new host. These ***immune evasion*** strategies can take several forms. Large DNA viruses, including pox viruses and herpesvirus, encode proteins that mimic host cytokines, cytokine receptors, or cell death regulators to limit the host antiviral and inflammatory response.[15,346,459,512] Most RNA viruses that cause disease in mammalian hosts encode proteins that modulate aspects of the host type I interferon response either by antagonizing specific components of the interferon induction/signaling network or through generalized shutoff of host protein synthesis, which effectively limits the production of antiviral effector molecules.[5,6,148,149] Lentiviruses also encode immune modulatory proteins that antagonize specific intrinsic restriction factors, such as the HIV accessory protein Vif, which mediates inhibition of the cellular restriction factor APOBEC3G.[306,435] Due to the limited coding capacity of RNA viruses, many immune evasion activities are performed

by multifunctional proteins that may modulate multiple aspects of the host response (eg, influenza A virus NS1 protein) or even play essential roles in viral replication, such as the alphavirus nsP2 protein that mediates host cell shutoff of host macromolecular synthesis, modulates type I IFN signaling, and is also an essential component of the viral replication complex.[5,6,148,149] Although most of the known mediators of viral immune evasion are virally encoded proteins, there is a growing recognition that noncoding RNA elements of the genome can also modulate the host response, as illustrated by a stem loop structure on the extreme 5′ end of the alphavirus genomic RNA that allows virulent alphaviruses to avoid inhibition of viral protein synthesis by the host IFIT1 protein.[209,210] During acute infection, viral immune evasion strategies commonly focus on the host innate immune response, while evasion of adaptive immunity is more important for maintaining chronic infection. Therefore, viruses that cause chronic infection often employ mechanisms for evading antiviral T- and B-cell responses. These include limiting viral gene expression during latency (eg, herpesviruses), the rapid generation of viral mutants to outrun adaptive immunity (eg, HIV or HCV), or interfering with host cell antigen presentation to avoid detection by antiviral T cells (eg, herpesviruses).[261,423]

Tropism

Tropism is the capacity of a virus to infect specific cells, tissues, or species. Tropism is a fundamentally important contributor to viral pathogenesis and virulence as the capacity to induce disease is dependent on the cells and tissues infected. For example, a neurotropic virus such as West Nile virus (WNV) can cause encephalitis or paralysis while a virus with tropism for CD4 T cells such as HIV causes immunodeficiency.

Viral interactions with entry receptors are key determinants of tropism and are the major determinants for cell tropism and host range for numerous viruses (eg, coronaviruses). However, there are many additional factors that determine cell, tissue, and species tropism, including expression of essential proviral factors, the absence of restriction factors, or the virus's ability to evade these restriction factors. For example, the counteraction of viral restriction factors by accessory proteins encoded in primate lentiviruses occurs in a species-specific manner and dictates cross-species transmission of these viruses.[124] Other factors, such as cellular differentiation state, can affect a virus's ability to replicate, as illustrated by the impact of cellular differentiation on HPV replication (Fig. 8.3). The comprehensive identification of both proviral and antiviral factors and the role that they play in either promoting or limiting the replication of specific viruses has provided important new insights into the regulation of cell tropism for a number of medically important human viruses.

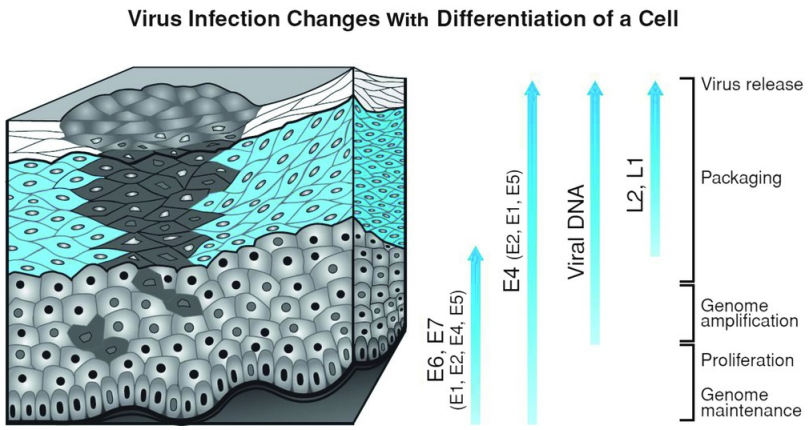

FIGURE 8.3 **Cellular differentiation plays a critical role in papillomavirus pathogenesis**. Shown are the relationships between stages in cell differentiation on the replication of a papillomavirus on the far right, the nature of the progressions of infection through the layers of the skin on the left, and the patterns of papillomavirus gene expression as shown by the labeled *blue arrows* in the center. Virus-infected cells in the skin are designated in *dark gray*. (This figure was adapted by H.W. Virgin IV from Doorbar J. The papillomavirus life cycle. *J Clin Virol*. 2005;32:S7-S15. Copyright © 2005 Elsevier. With permission.)

Virulence Determinants

Virulence is determined by the capacity of a virus to grow, be invasive, infect vulnerable cells, evade the immune system, subvert cellular processes, and cause tissue damage. While virus extrinsic factors, such as mosquito saliva–mediated enhancement of arbovirus infection,[430] or the immune-competence of the infected host can affect virulence, virally encoded factors play a major role in determining whether a given virus is virulent. It is well established that viral genotype has a major impact on virulence,[20,103,121,129,204,235,303,311,462,488-490,492] and the availability of molecular clone technologies, and recent advances in synthetic biology have allowed for the rapid generation of molecularly cloned viruses and the identification of viral genetic determinants of virulence.[235,294,319,320,380,382,488-490] Subsequent studies have shown that different viral genes can confer capacities for infection in different tissues, indicating that viral virulence genes can be tissue specific,[189,310] and viral variants that arise during chronic infection are often cell and tissue specific.[2,3,188,245,338]

A ***virulence determinant*** is a virally encoded feature that contributes to viral virulence and where variation in that determinant affects the virulence of related viruses. Since viral replication is generally required for a virus to induce disease, any viral gene (or noncoding RNA element) that is essential for viral replication contributes to virulence, and variants in a gene that affects viral replication can be virulence determinants. Replication independent functions, such as immune evasion or alterations in cell tropism, can also affect viral virulence, and viral variants that affect these activities are considered to be virulence determinants. Genes that are not essential for viral replication, which are also referred to as ***accessory genes***, are often required for *in vivo* virulence and can therefore also act as virulence determinants. There are numerous examples of accessory proteins acting as virulence determinants, including the HSV γ34.5 gene, which is dispensable for viral replication, but which is required for virulence due to its role in modulating multiple aspects of the host antiviral response.[104,307,362] While large DNA viruses such as herpesviruses and pox viruses encode large numbers of virulence-associated accessory genes due to their large genome size, RNA viruses and retro/lentiviruses also encode accessory proteins that contribute to viral virulence. Examples include the previously mentioned HIV Vif protein that is dispensable for viral replication in cells lacking APOBEC3G but is essential for counteracting the antiviral effects of APOBEC3G,[306,435] as

well as the NS1 protein of influenza virus, which is essential for viral virulence in animals with an intact type I interferon system.[152,522] However, it is also important to remember that many viral proteins are multifunctional, and proteins that may be essential for viral replication, such as the alphavirus nsP2 protein, may also regulate aspects of the host innate immune response or modulate cellular functions.[5,149,159,321] Therefore, it is sometimes difficult to separate out these essential enzymatic processes from the accessory functions of these proteins, and it is important to recognize that allelic variations in the function of essential genes can be important determinants of viral virulence. In this case, the specific property associated with a given alleles effect on virulence is considered a virulence determinant. For example, allelic variants in the PB2 protein, which is an essential component of the H5N1 avian influenza virus RdRp, regulate viral host range, are associated with enhanced replication in mammalian cells, affect viral spread in tissues, and promote enhanced *in vivo* virulence in mouse models of virus-induced disease.[192,284,444,470]

Virulence Genes and Determinants May Not Encode Proteins

While virulence is often mediated by viral proteins, noncoding promoters or elements in the viral genome can also regulate viral virulence. For example, EBV infection is associated with the development of B-cell lymphomas. The EBV EBNA1 protein, which plays an important role in the maintenance of latent EBV infection, can be expressed from four different promoters termed Cp, Wp, Fp, and Qp, each of which is regulated in distinct ways by viral and host proteins.[461] These finely tuned promoters, which ensure appropriate EBNA1 expression and regulation in B cells, are therefore properly considered virulence determinants for EBV-associated malignancies. Another example of nonprotein coding virulence determinants are virally encoded microRNAs, which are expressed by a number of mammalian viruses, including herpesviruses, adenoviruses, and polyomaviruses.[169,293,453] These noncoding RNAs target multiple cellular processes, including cell cycle regulators, apoptotic machinery, or virus-encoded transcripts and play a role in promoting viral persistence. An interesting example are the tRNA-like noncoding RNAs found in the left end of the murine gammaherpes virus γHV68 genome.[57] Remarkably, these tRNA-like RNAs are cotranscribed with viral miRNAs to generate tRNA-miRNA-encoded RNAs or TMERs[115] (Fig. 8.4) and TMER-deficient γHV68 strains are attenuated *in vivo*.[116,134] Noncoding functions within RNA virus genomes can also contribute to virulence. Mutations within a stem loop structure found in virulent alphavirus strains are associated with increased sensitivity to IFIT1 (Fig. 8.4), and viruses encoding these mutations are attenuated in their ability to replicate and cause disease *in vivo*.[209,210] The 3′ UTR of flavivirus genomes contain numerous RNA structural elements including a class of structured RNAs termed exoribonuclease-resistant RNAs (xrRNAs) that have recently been identified in a broad range of animal and plant viruses[504] (Fig. 8.4). During flavivirus replication, xrRNAs resist degradation by the 5′-to-3′ exoribonuclease Xrn1 due to the presence of a ringlike structure through which the 5′ end of the RNA threads.[7,82,225] XrRNA-mediated inhibition of Xrn1 results in the production of flavivirus subgenomic RNAs (sfRNAs) that are derived from the 3′ end of the viral genome.[377] These sfRNAs have been shown to modulate multiple aspects of the host response, including the siRNA and type I IFN response.[455] Mutations that prevent the generation of sfRNAs have been shown to be attenuating for multiple pathogenic flaviviruses, and this subgenomic RNA is considered to be a flavivirus virulence determinant.[455] Noncoding RNAs can also affect cell tropism, as illustrated by EEEV, where microRNA target sites in the viral 3′ UTR that are targeted by miR-142-3p prevent the virus from replicating in myeloid cells, limiting detection by the host immune system and promoting viral neuroinvasion and disease.[485]

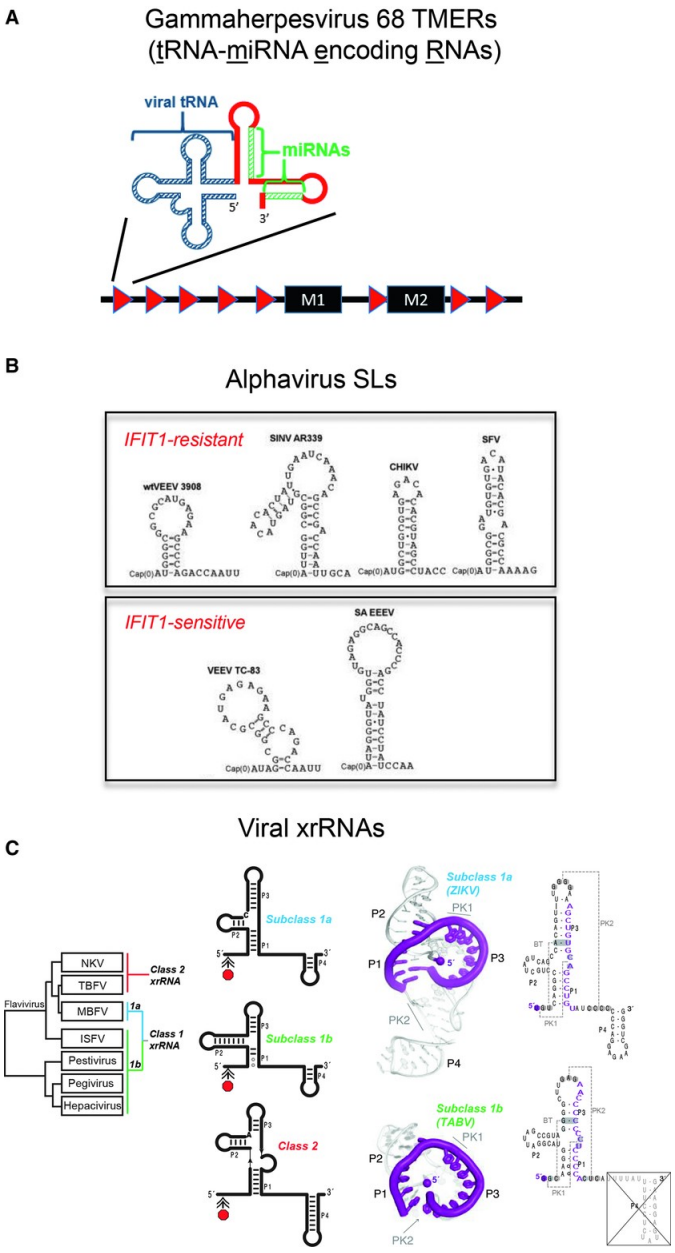

FIGURE 8.4 **Examples of viral noncoding RNAs that influence viral pathogenesis**. **A**. Schematic of tRNA-miRNA encoding RNAs (TMERs) found in the murine gammaherpesvirus 68 genome. These TMERs promote viral pathogenicity through both the tRNA and miRNA elements. (From Diebel KW, Claypool DJ, van Dyk LF. A conserved RNA polymerase III promoter required for gammaherpesvirus TMER transcription and microRNA processing. *Gene*. 2014;544:8-18. doi:10.1016/j.gene.2014.04.026; images courtesy of Dr. Linda van Dyk.) **B**. Schematic of stem loops found in the 5′ UTR of the RNA genome of alphaviruses that are associated with resistance or susceptibility to IFIT1-mediated restriction. (Adapted from Reynaud JM, Kim DY, Atasheva S. IFIT1 differentially interferes with translation and replication of alphavirus genomes and promotes induction of type I interferon. *PLoS Pathogens*. 2015;11:e1004863; courtesy of Drs. Ilya Frolov and Michael Diamond.) **C**.*Left*: Genera in the Flavivirus supergroup encode one of three subclasses of xrRNAs: 1a, 1b, or 2. *Middle*: Ringlike structures (*purple*) that confer resistance to Xrn1 degradation are found in xrRNAs of each subclass despite differences in primary sequence. *Right*: Schematic of the secondary structure of subclass 1a and 2 xrRNAs. PK, pseudoknot; BT, base triple. (Adapted from Vicens Q, Kieft JS. Shared properties and singularities of exoribonuclease-resistant RNAs in viruses. *Comput Struct Biotechnol J*. 2021;19:4373-4380. Copyright © 2021 Elsevier. With permission.)

The Study of Viral Pathogenesis

The study of viral pathogenesis takes advantage of a scientific tool kit that includes standard assays for quantifying infectious virus, measuring virus-induced pathology in infected tissues, and measuring virus-induced disease. However, recent advances in molecular biology, as well as areas such as synthetic biology, genomics, tissue engineering, imaging, and animal models have enhanced investigators' ability to probe the specific viral and host factors that impact virus-induced disease. Understanding these tools and how they can be applied to viral pathogenesis research is essential for anyone interested in viral pathogenesis or viral pathogenesis research. In this section, we will discuss some key examples of the types of research tools that are employed in viral pathogenesis research.

Epidemiology

Epidemiology plays an important role in understanding the pathogenesis of most viruses. Epidemiology research often plays an essential role in linking specific viruses to disease outbreaks, or even specific disease manifestations, while identifying disease patterns and characterizing mechanisms of viral transmission. Epidemiology studies can also provide insight into factors that put individuals at risk of developing disease, including prior immunity, comorbidities, and demographic factors (eg, age, gender), and this information provides strategies for reducing viral spread or protecting individuals who are at highest risk for developing disease. This information can then inform which questions are pursued by viral pathogenesis researchers to mechanistically understand how these factors contribute to the pathogenesis of a specific virus. The importance of epidemiology research for guiding viral pathogenesis can be illustrated by the following examples.

Kaposi sarcoma (KS) was recognized as a common manifestation of HIV-induced acquired immune deficiency syndrome (AIDS).[78] However, epidemiology work indicated that HIV status did not accurately predict risk of developing KS, suggesting that other factors contributed to KS risk.[39] The discovery of Kaposi sarcoma herpesvirus (KSHV)[79] was followed by additional epidemiologic studies, which provided additional evidence linking KSHV to KS.[39,151,329] These discoveries spurred numerous studies focused on understanding the pathogenesis of KS, as well as other KSHV-associated cancers, and understanding how KSHV interacts with the host to promote tumor development.

A second example of the importance of epidemiology in pathogenesis research is provided by ZIKV, which was discovered in 1947 in Uganda.[114] Although initially considered to be largely innocuous, during a series of outbreaks starting in 2006, the virus was associated with febrile illness and arthralgia.[123] ZIKV infection was also associated with an increase in the incidence of Guillain-Barre syndrome, a neurologic disease of peripheral nerves that can result in ascending paralysis and even respiratory failure.[72] The capacity of ZIKV to cause serious disease was further illustrated by a massive ZIKV outbreak in South America that occurred in 2015. During this outbreak, a cluster of cases of fetal microcephaly was noted in Northern Brazil, and subsequent epidemiologic studies demonstrated a strong association between ZIKV seropositivity and fetal birth defects.[60,99] Follow-up studies found that infection early in pregnancy was associated with increased risk of congenital birth defects.[290,502] This association between congenital ZIKV infection and the development of birth defects sparked a significant amount of research focused on understanding the pathogenesis of ZIKV-associated birth defects. These included studies focused on developing small animal and nonhuman primate models of ZIKV infection[59,196] and using these models to understand how ZIKV crosses the placental barrier and causes disease. Other topics of investigation have included the identification of viral virulence determinants[24,73,97,283,341,388,439,440,460,540] and elucidating host factors that play either protective or pathologic roles during viral infection.[76,221,351,486] While the pathogenesis of ZIKV-induced congenital birth defects is still not completely understood, the rapid pace of this research is in part due to the guidance provided by early epidemiologic studies that defined key parameters associated with the disease.

Clinical Research

Clinical virus disease research, which involves the study of control and infected patients or samples derived from those patients, also plays a key role in understanding the pathogenesis of viruses that cause disease in humans. Historically, clinical observations have involved the careful documentation of disease signs, the use of diagnostic markers such as liver enzyme levels and white blood cell counts/composition in the blood, characterization of viral isolates, and analysis of tissue biopsies or autopsy specimens. This information has been essential for defining key aspects of virus-induced disease and guiding the direction of basic science approaches for understanding the pathogenesis of virus-induced disease. However, with advances in molecular diagnostics, including methods for systematically measuring host gene expression (eg, RNAseq), as well as high-resolution *in vivo* imaging techniques, such as magnetic resonance imaging (MRI), it is now possible to obtain a more comprehensive picture of the molecular signatures associated with the response to specific classes of viral pathogens or specific disease outcomes.

One of the major limitations of studying clinical outcomes during natural infection in humans is the difficulty in controlling for variables that can affect disease outcome, such as viral dose, viral genotype, time post exposure, and underlying health conditions. While it is difficult to control for these variables during natural human infection, controlled human challenge studies using defined viral challenge stocks with close monitoring of virus-induced disease have provided important insights into virus-induced disease processes. While these types of studies are often restricted to viruses that cause self-limited or mild disease, they still have the potential to provide important insights into how these viruses cause disease. For example, human challenge studies with seasonal influenza A virus have provided information on the disease course following exposure,[315,316] the impact of biological sex on disease outcome,[166] and correlates of immune protection[205,530] and have been used to study viral transmission.[243,345] Likewise, human norovirus challenge studies have provided information on the impact of the microbiome on susceptibility to norovirus-induced disease,[369] and for studying the role of blood histo-group antigen secretor status on susceptibility to virus-induced disease.[144] This information, along with the molecular insights provided by more advanced readouts of virus-induced gene expression, metabolic changes, and imaging of tissues from infected patients can then be used to draw conclusions about how viruses or the host response affect specific aspects of the disease process, while also guiding downstream pathogenesis studies using animal models or cell culture systems (see below) to further define the mechanisms driving the pathogenesis of virus-induced disease.

Cell Culture Systems

The development of methods for culturing mammalian cells *in vitro* represents one of the most significant advances in the history of the virology field. Prior to the availability of continuous cell lines, maintenance and detection of disease-associated viruses relied on passage of the virus through permissive animal models. However, the development of cell culture enabled the generation of high-titer virus stocks and methods for quantifying viral titers (eg, plaque assays and tissue culture infectious dose [TCID50] assays) based on the seminal work of Renato Dulbecco.[125] These techniques revolutionized the virology field and are now routinely used for growing, quantifying, and characterizing a wide variety of mammalian viruses and even viruses affecting other species. Furthermore, these techniques have been essential for the development and production of live-attenuated and inactivated vaccines such as the polio vaccine.[26,128,416]

While continuous cell lines are an essential tool for modern virology, these cell lines have limitations. Many cell lines lack key innate immune system components, such as an intact type I IFN system,[111,113,363] and the immortalization process used to generate continuous cells often results in changes in cell cycle regulation or other metabolic processes that make these cells quite different from normal cell types. Therefore, the virology field often utilizes nontransformed cells or even primary cells such as primary fibroblasts, neurons, airway epithelial cells, or dendritic cells[29,87,150,381] to more closely model viral interactions with the infected host. However, even primary cell cultures fail to reproduce the complex interactions that occur within virally infected organs. Therefore, the development of organoid cultures, which better mimic the

architecture and cellular interactions that occur within intact organs, provide the field with new systems for studying how viruses interact with infected organs *ex vivo*. These types of systems have already been used to model viral infection for enteric, pulmonary, and neurologic pathogens.[49]

Viral Molecular Clone Technologies

The ability to manipulate viral genomes using infectious clone technologies has become an important tool for identifying and characterizing viral virulence determinants. As noted above, cell culture methods have enabled the production, maintenance, and study of a wide variety of viruses. Prior to the modern molecular biology era, studies using reassortment of segmented viruses such as reovirus and influenza viruses allowed investigators to link specific pathogenesis phenotypes (eg, viral spread or transmissibility) to specific viral genes[240,471,492] while the use of mutagens, the development of temperature-sensitive mutants, and complementation studies allowed viral enzymatic processes or functions to be attributed to specific virus genes in a wide variety of DNA and RNA viruses including HSV,[426,427] alphaviruses,[69,308,429] and picornaviruses.[386] However, the development of recombinant DNA technology[96,218] greatly facilitated the virology field's ability to identify and molecularly characterize the specific genetic determinants that affect virulence, or prove the causality of a gene for a specific phenotype through deletion or mutagenesis of that gene. Reverse genetics technologies, which include using recombination to generate DNA viruses with mutations in specific genes,[457] as well as infectious cDNA clone technology, where the viral genome is introduced into a DNA molecule (eg, plasmids or bacterial artificial chromosome) that is then used to launch the production of infectious virus, allowed for the targeted manipulation of viral genomes. These tools then allowed investigators to use standard molecular biology approaches to mutagenize specific regions or introduce specific nucleotide substitutions into the virus. For RNA viruses, infectious clone technology was initially developed using bacteriophage.[475] The technology was then used to study molecular determinants of replication and pathogenesis for positive sense RNA viruses such as poliovirus or alphaviruses due to the infectious nature of their RNA genomes.[382,392,400] However, infectious clone technology has now been extended to all of the major classes of viruses, including DNA viruses,[301,322] retroviruses,[299] negative sense RNA viruses,[14,64,274,344,431,526] and double-stranded RNA viruses.[253] Therefore, it is possible to use this technology to genetically manipulate almost every pathogenic virus and to use these techniques to test the role of specific viral genes or even single nucleotide variants in regulating different stages of the viral infection process or their role in disease pathogenesis. Moreover, these technologies also facilitate broad genetic approaches including deep mutational scanning and the use of bar-coded viral libraries to track and determine the influence of viral diversity in viral pathogenesis.[47,498]

Synthetic biology, where DNA molecules are chemically synthesized, allowing for the *de novo* generation of DNA clones without access to physical viral genomes as templates, has also proven useful for the generation and manipulation of viral infectious clones. This technology was initially used to generate an infectious clone for poliovirus[77] but is now routinely used to generate clones for a wide variety of viruses.[203,332,436] Since the *de novo* synthesis of viral genomes requires only sequence information, this approach has allowed for the rapid generation of infectious clones of newly discovered viruses, or even for viruses where the viral genome sequence has been identified through field studies but the actual virus has not been isolated.[319] These synthesized viruses have been used to test whether zoonotic coronaviruses have the capacity to replicate in human cells and thus have the potential to infect and cause disease in humans.[319] Furthermore, these synthetic biology approaches can be used to generate large numbers of viral mutants, allowing investigators to perform saturation mutagenesis studies across regions of the viral genome to identify potential viral virulence determinants or functional domains in viral proteins.[71,464,465] Therefore, synthetic biology provides a powerful set of tools for manipulating viral genomes and the identification and study of virally encoded virulence determinants.

Functional Genomics Technologies

The host response to viral infection plays a major role in determining whether an individual is susceptible to virus-induced disease, or even what type of disease an individual develops. For example, signaling by NF-kB plays a major role in regulating which organ systems are affected by reovirus infection, where deficiency in the nfkb1/p50 gene results in reduced virus-induced apoptosis in the CNS but an increase in viral replication and damage in the hearts of reovirus-infected mice.[352] Therefore, understanding how individuals respond to viral infection at the molecular level can provide important information on the gene expression profiles that are associated with specific disease outcomes or how responses differ when individuals are infected with viruses of differing virulence. The application of functional genomics approaches, which span a wide variety of methods for analyzing host genes or gene products, has had a major impact on our understanding of viral pathogenesis. Given the breadth and rapid developmental pace of this field, we will focus on a few key examples of how these approaches can be used to study how viruses interact with the host, or to determine the functional impact of specific host gene products on viral infection.

Prior to the availability of whole genome sequence information, most analysis of virus-induced host gene expression was limited to analyzing a limited number of host genes or gene products using targeted assays such as Northern blot, reverse-transcriptase polymerase chain reaction (RT-PCR), ELISA, or protein-specific bioassays. However, the availability of whole genome sequence information for humans[215,501] and key laboratory species, such as the mouse,[90,239,336] combined with the development of high throughput platforms for measuring host gene expression, such as microarrays and high throughput RNA sequencing technologies, made it possible to measure genome wide changes in host gene expression.[68,523] These technologies have enabled investigators to address a wide variety of questions, including the study of how host cells or tissues respond to specific viral infections, compare the host response to different viral pathogens that target the same tissue,[317,318] and analyze the role of specific viral proteins in modulating host transcriptional responses.[449] More recent advances, including the development of single cell RNA sequencing technologies, and methods for measuring host gene or protein expression *in situ*,[206,484] such as spatial transcriptomics and mass cytometry, allow investigators to differentiate responses of virally infected vs uninfected cells, account for heterogenous responses during an infection, or analyze the response of specific immune or nonimmune cells in an infected tissue.

High throughput analysis of virus-induced changes in transcriptional responses, protein expression, or other metabolic processes provides important information on how the host or even specific cell types respond to a given viral infection. This information can then be used to test what role specific host genes or gene products with altered expression play in the virus-induced disease process. However, in addition to these gene-by-gene approaches, the virology field has also taken advantage of high throughput methods for interrogating the role of entire classes of genes or even across the entire genome for their role as pro- or antiviral factors. These screens can be generally divided into two categories involving either the overexpression of libraries of genes or the knockdown or knockout of host genes to test their role in viral replication. Overexpression libraries have proven useful for testing the role of specific genes in inhibiting replication (antiviral factors) against a variety of viruses.[234,266,432,434] The development of multiplexed, barcoded short interfering RNA (siRNA) libraries for knocking down the expression of

thousands of host genes[43,367] provided an opportunity to functionally test genes across the entire genome for their role in promoting or antagonizing replication by a wide variety of viruses, including early studies with HIV, flaviviruses, and influenza virus.[61,191,197,259,262,473,548] The availability of high throughput CRISPR-based methods for knocking out, knocking down, or even upregulating gene expression has provided additional tools for identifying host genes with antiviral or proviral activity.[89] While the number of viruses studied using these methods are too numerous to discuss in this chapter, these approaches have and continue to provide important insights into the role of specific host genes in every stage of the viral life cycle, including the identification of host genes that regulate viral tropism or host range. For example, siRNA and CRISPR based screens have identified a number of entry receptors for different alphaviruses,[94,302,408,544] and these results have set the stage for downstream *in vivo* studies evaluating how these molecules affect viral cell tropism or host range.[244,543]

High throughput analysis of host gene function (eg, knockdown or knockout screens) in cell culture has been useful for identifying genes that act at the cellular level. However, many genes impact viral pathogenesis at the systemic level through regulation of complex biologic processes that cannot be easily replicated in cell culture, such as complex interactions regulating the systemic immune response. Whole organism high throughput genetic screens can be readily performed in genetically tractable organisms with rapid reproduction times such as the roundworm *Caenorhabditis elegans*,[231] the fruit fly *Drosophila melanogaster*,[231] and the zebrafish *Danio rerio*,[421] and these systems have been useful for identifying and characterizing host genes that regulate the replication and pathogenesis of viruses that naturally infect those species or related organisms as well as for human pathogens.[23,228,410,472] However, while nonmammalian models have provided important insights into genetic factors that regulate virus-induced disease, those models do not recapitulate many of the organ systems or complex immune regulation in mammals and therefore cannot model more complex virus/host interactions that occur in humans. While homologous recombination and enzyme-based genome editing methods (eg, CRISPR) make it relatively easy to knock out or modify mammalian genes *in vivo* using laboratory mice,[4,257] high throughput genome-wide whole organism genetic screens are more difficult in mammalian species due to longer reproduction times and challenges associated with knocking out or knocking down genes in the whole animal, as well as the logistical challenges and costs associated with conducting virus susceptibility assays in the thousands of animals required for such a screen. Therefore, the majority of *in vivo* studies have focused on the characterization of individual host genes or small sets of genes in virus-induced disease using gene-specific knockout mice. However, approaches such as *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis of the whole genome have been used to screen for genes that regulate virus susceptibility,[101,532] and the availability of large libraries of gene-specific mutant mice generated by homologous recombination, CRISPR gene editing, or ENU mutagenesis[19,454,496,519] makes it possible to screen large numbers of host genes for their impact of viral pathogenesis using mouse models.

Animal Models

Animal models are another important set of tools for understanding viral pathogenesis. As noted above, human clinical studies can provide insights into multiple aspects of pathogenesis. However, human infection outcomes can be influenced by a variety of factors, including viral dose, exposure route, and underlying medical conditions that can be difficult to control for during natural infection. While tightly controlled human challenge studies can account for many of these variables, due to safety concerns, these types of studies are generally limited to viruses that cause mild or self-limited disease and are conducted in healthy individuals lacking many of the risk factors that might lead to poor disease outcomes during natural infection. Furthermore, the types of highly invasive studies needed to study virus-induced pathology or define the role of specific host factors in disease pathogenesis are often not possible in humans. Therefore, animal models, which allow invasive experiments and where it is feasible to control many of the experimental variables that confound human studies, have been an essential tool for studying viral pathogenesis. Animal models of virus-induced disease can make use of either nonhuman viruses that are related to pathogenic human viruses, but which naturally infect an animal host, or human viruses that are capable of infecting and causing disease in a permissive animal species. Both of these approaches have provided important information on viral pathogenesis in general and have informed our understanding of how viruses cause disease in humans.

Natural Infection Models of Viral Pathogenesis

Natural infection models are particularly useful for *in vivo* pathogenesis studies when the analogous human pathogen is restricted to replicating in human cells. Examples of natural animal pathogens as models of human disease include simian immunodeficiency virus (SIV) and murine cytomegalovirus (MCMV).[142,323] There are multiple SIV strains circulating in old world nonhuman primate (NHP) species, and phylogenetic studies indicate that both HIV-1 and HIV-2, the causes of AIDS, originated from SIV strains that were transmitted to humans through zoonotic spread.[200,207,370,442] While SIV strains often cause little disease when infecting their natural NHP hosts, infection of a different NHP species can result in disease. For example, SIV derived from sooty mangabeys causes AIDS-like disease in rhesus macaques,[242] and this model has been important for studying the pathogenesis of AIDS and as a resource for the development and testing of vaccines and antivirals against HIV-1 and HIV-2.[323]

Human cytomegalovirus (HCMV) latently infects a large fraction of the human population without causing significant disease but is a major cause of birth defects when primary infection occurs during pregnancy and also causes severe disease in immune compromised individuals.[179] Cytomegaloviruses (CMV) are generally species specific, and while HCMV can be studied in humanized mouse models,[515] these models do not yet fully recapitulate viral pathogenesis or interactions with the host immune system. Therefore, the majority of studies of cytomegalovirus pathogenesis have used CMV strains that naturally infect rodents (eg, MCMV or rat CMV) or NHPs (rhesus CMV).[142,384] MCMV, which is a natural mouse pathogen, has allowed investigators to take advantage of the wide variety of genetic and immunology tools that exist for working with laboratory mice to study MCMV pathogenesis. Although there are important differences between HCMV and MCMV in terms of their genome organization/gene conservation and specific aspects of their pathogenesis, such as congenital infection,[142] MCMV has been an important model for studying specific HCMV-induced disease states, such as HCMV-associated cardiovascular disease.[50] The MCMV model has also provided important insights into mechanisms regulating immune control of acute infection and maintenance of viral latency,[13,102,195,300] and for understanding how virally encoded genes modulate immune cell populations.[132]

Human Viruses in Animal Models

While infection models with natural animal pathogens are powerful tools for studying viral pathogenesis, the viruses used in these models are often genetically distant from the analogous human viruses and may have evolved to interact with the infected host in ways that are significantly

different from the human virus. Therefore, in cases where the actual human pathogen is able to replicate and cause disease in a relevant animal model, it is often preferable to utilize the human virus. While there are often important differences between the pathogenesis of these viruses in animals compared to human infection, these model systems have provided important insights into the pathogenesis of a wide variety of human pathogens, including Ebola virus, influenza A virus, SARS-CoV-2, HSV, and VEEV.[422]

Some human viruses, such as Ebola virus, HSV, and VEEV can replicate and cause disease in animal models without adaptation, allowing human clinical isolates to be directly evaluated in models such as laboratory mice or nonhuman primates. For example, clinical isolates of Ebola virus causes disease in macaques that is very similar to human disease, including disseminated intravascular coagulation,[162-164] and these models have been important for the development and testing of vaccines and therapies against filoviruses.[162-164,226] However, not all human viruses can directly replicate and cause disease in animals, and several strategies have been used to overcome this problem.

1. If a virus is able to replicate in the animal, but is attenuated or does not cause disease, it is often possible to adapt the virus through repeated passage to generate an animal adapted virus that replicates efficiently in the animal host and causes disease signs that are similar to those seen in humans. These approaches have been used to generate mouse-adapted influenza A virus strains, SARS-CoV-1 and SARS-CoV-2 strains, and Ebola virus strains that are capable of causing disease in mice.[56,63,230,402]

2. While blind passage is often used to generate mouse-adapted viruses, if the specific determinants of host range are known, it is also possible to engineer viruses to permit replication in an animal host. Early pandemic strains of SARS-CoV-2, which utilizes ACE2 as a cellular receptor, are unable to utilize mouse ACE2 and are therefore unable to infect or cause disease in mice.[117] Mutating two amino acid positions in the viral spike protein confers the virus with the ability to utilize mouse ACE2, and this virus replicates efficiently in the lungs of wild-type mice.[117] However, this virus was nonpathogenic in mice, and additional adaptive mutations generated through serial passage were needed to confer virulence and generate a mouse model reproducing SARS-CoV-2 disease signs similar to those observed in SARS-CoV-2 infected humans.[277]

3. Some viruses are unable to replicate in animal hosts due to the lack of appropriate cell entry receptors, and the transgenic expression of the human receptor either in the germline or through the transient delivery of the receptor through a viral expression vector can result in the development of a permissive model of virus-induced disease. These approaches have been used to generate mouse models for viruses such as polio virus, measles virus, MERS-CoV, and SARS-CoV-2.[256,438,546,547] It is also possible to either replace the mouse gene with the functional human receptor by homologous recombination or use CRISPR-mediated gene editing to introduce specific coding changes into the mouse gene to create a functional, humanized, viral receptor.[95,282,368] While transgenic or gene knock in mice is commonly used to express functional viral receptors, these approaches can also be used to modify host genes that regulate aspects of the host immune response, such as the type I interferon system, that act as viral restriction factors. For example, the NS5 protein of ZIKV promotes proteasomal degradation of human STAT2 to antagonize the host type I IFN system,[168] but ZIKV does not antagonize mouse STAT2, which restricts the virus's ability to replicate efficiently and cause disease in immunocompetent mice. However, replacement of murine STAT2 with human STAT2 resulted in a mouse model where a mouse adapted ZIKV strain was able to replicate to higher levels in the placenta and more efficiently cause fetal infection compared to wild-type mice, thereby generating an immunocompetent mouse model of ZIKV transplacental infection.[168]

4. Some viruses are unable to replicate in nonhuman hosts due to multiple incompatibilities or restriction factors that make it difficult to generate animal models by genetically engineering the virus and/or the host genome. For viruses such as HCMV, HIV, HCV, and varicella-zoster virus that are restricted to humans, mice carrying allografts of the human immune system, and/or specific organ systems have provided useful models for studying the pathogenesis of these viruses.[27,160,276,424,515,541]

5. For some human restricted viruses, such as hepatitis B virus (HBV), it is also possible to express the viral genome as a transgene, which leads to production of infection virus in specific cell types.[182,184] This system has provided important insights into the role of specific components of the host immune system in clearing HBV infection and driving virus-induced immune pathology.[183]

Selection of Appropriate Animal Models

Despite their importance for viral pathogenesis research, it is important to remember that all animal models have limitations and no model completely recapitulates all aspects of virus-induced disease in humans. Furthermore, different model systems have strengths and weaknesses, both for modeling specific aspects of virus-induced disease and for mechanistically studying the viral and host factors that contribute to virus-induced disease. For example, of the commonly used vertebrate animal models, NHPs are most closely related to humans, and therefore share similarities in both their physiology and genetics, which often leads to disease outcomes that more closely reflect those seen in humans. For this reason, NHPs are considered to be the gold standard for studying the pathogenesis of several important human pathogens, including filoviruses (eg, Ebola virus).[452] However, NHPs also have several limitations, including high cost, the need for specialized facilities, limited availability, and a lack of robust genetic tools for studying the role of specific host genes in disease pathogenesis. Therefore, a variety of other models for studying filovirus pathogenesis have been developed, including hamsters, guinea pigs, and mice.[63,98,127,452] Although these models require the use of adapted viruses,[63,98,127,452] or the use of type I IFN deficient animals,[62] they do recapitulate key aspects of the virus-induced disease process and have been useful systems for studying filovirus tissue tropism or performing detailed studies of virus-induced pathology.[452] Furthermore, mouse models provide access to a wide range of genetic tools, including gene-specific knockout mice and genetically diverse reference populations (eg, the Collaborative Cross [CC]) that can be used to study the role of specific host genes or host genetic variation in the pathogenesis of filovirus-induced disease.[395,452] These small animal models can then be viewed as complementary tools that can be used to address questions that either cannot be readily addressed in NHPs, such as the role of specific host genes in virus-induced disease susceptibility, or as low-cost alternatives that can be used for initial discovery studies where results are confirmed using NHPs or human data sets. As we will discuss in more detail below, it is important to weigh the strengths and weaknesses of any animal model when considering how well it models virus-induced disease, whether that model can provide useful information on the pathogenesis of a given infection, or whether it might be necessary to use different model systems to address various aspects of the virus-induced disease process.

Selection of an animal model for studying virus-induced disease is partly driven by how well that model reproduces the virus-induced disease process; however as noted in the previous paragraph, factors such as ease of use, cost, availability, experimental tractability, and the availability of research reagents also influence model selection. For these reasons, the laboratory mouse, which fulfills all of these criteria, has played a dominant role in studying the pathogenesis of a wide variety of viruses[309] (Fig. 8.5). While all of these factors contribute to the popularity of the mouse as a model system, the large panel of genetic tools in the mouse, including inbred mouse strains where every mouse in a strain is

genetically identical, and the ability to easily knock out or modify specific genes in the mouse genome, has made the mouse a particularly powerful resource for understanding how viruses interact with the host.[422] Inbred mouse strains allow investigators to use large numbers of genetically identical mice for studying the response to specific viruses, allowing investigators to control for host genetic effects when studying how other viral or host factors, such as viral genetics, viral dose, microbiome, prior immunity, or environmental influences affect virus-induced disease. Knowledge of the whole genome sequence of these strains combined with gene knockout and gene editing technologies (eg, CRISPR gene editing), has allowed investigators to knock out or modify specific host genes while controlling for genetic effects across the rest of the genome.[4,257] These technologies and the subsequent availability of large panels of gene-specific knockouts on defined genetic backgrounds allow investigators to interrogate the role of host genes in the pathogenesis of any virus that is capable of replicating and causing disease in the mouse.[19,454,496,519] In addition to allowing investigators to study the role of specific genes in viral pathogenesis, genetically modified mice have allowed for the development of new model systems. As discussed above, mice can be modified to express functional receptors or to ablate specific host antiviral factors to make mice permissive for a variety of viral pathogens.[95,168,256,282,368,438,496,546,547] Furthermore, knockout mice with severely compromised immune systems have been essential for developing humanized mice engrafted with human immune systems or specific tissues to generate small animal models for viruses that are only capable of replicating in human cells.[160,515] The dominance of the mouse as a model system, not just for virology but for biomedical science as a whole, has also led to the development of a wide array of reagents for studying biological processes in the mouse, including the immune system, thereby providing the virology community with a variety of tools for studying the host response to viral infection in the mouse.[422]

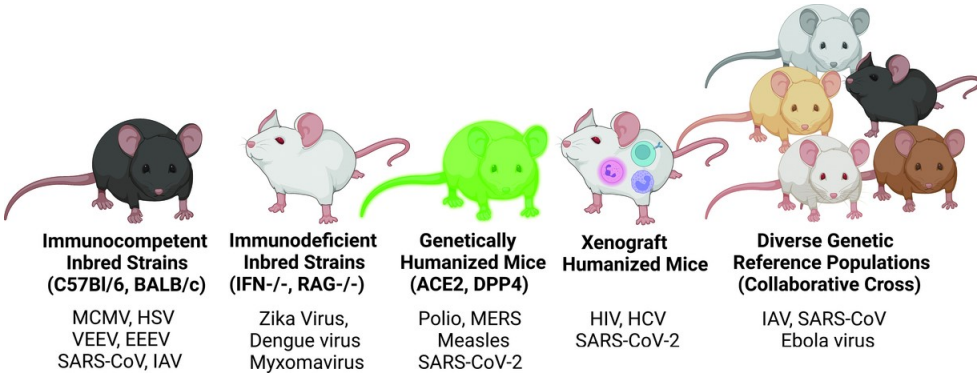

FIGURE 8.5 **Mouse models for viral pathogenesis studies**. Examples of different types of mouse models, and their utility for studying representative viruses discussed in this chapter. (Created with BioRender.com.)

It is important to consider the limitations of a model organism both when selecting a model and when interpreting data generated in that model. Using mice as an example, while mouse models have played a major role in the study of viral pathogenesis, they do have significant limitations. Mice are genetically distant from humans, and although many genes and gene functions are conserved between mice and humans, there are abundant examples of differences in specific genes or gene expression between mice and humans.[90,270,291,336] Therefore, it is important to keep these differences in mind when choosing a model system and if a nonconserved gene or pathway is being studied, it may be necessary to consider using more closely related species such as NHPs or humanize mouse models.

As detailed above, the use of inbred mouse strains has allowed virologists to control for host genetics in their studies; however, the reliance on a limited number of inbred mouse strains fails to account for impact of genetic diversity on virus-induced disease outcomes in humans.[348] This can be partly addressed by the use of genetically diverse inbred mouse populations, such as the CC mouse genetic reference population, which was derived from 8 founder strains and capture 90% of the genetic diversity in laboratory mice, while maintaining the advantages associated with fully inbred (eg, genetically reproducible) and genetically defined standard inbred mouse strains[348] (Fig. 8.5). The genetic diversity in the CC and the genetic reassortment of the original eight founder strain genomes resulting in new allele combinations across the CC strains often results in CC strains presenting with new phenotypes not seen in the original eight founder mouse strains.[348,463] The virology community has taken advantage of this aspect of the CC both to map host genes associated with variation in virus-induced disease[172,349] and to develop new models of virus-induced disease that more closely reflect disease outcomes in humans infected with viruses such as Ebola virus, SARS-CoV, and WNV.[140,171,172,258,395]

The physiology of a species can also have a major impact on whether that species should be used for studying the pathogenesis of a virus-induced disease or even specific aspects of the virus-induced disease process. For example, mouse models have been used extensively to study the pathogenesis of respiratory viruses such as influenza A virus (IAV), SARS-CoV, and SARS-CoV-2, and they have provided key insights into the role of specific host genes or immune cell populations in either limiting or exacerbating virus-induced disease.[56,250,277,481,547] However, mice do not efficiently transmit these viruses.[56,481] For this reason, transmission studies for influenza virus primarily rely on ferret or guinea pig models,[56,481] while SARS-CoV-2 transmission studies have predominantly used ferret or hamster models.[445,447] These types of differences may also be relevant to other aspects of virus-induced disease, such as congenital viral infection. HCMV is a common cause of congenital birth defects in humans,[179,401] but while the MCMV model has been useful for studying multiple aspects of CMV pathogenesis, this model is less useful for studying congenital infection in part due to structural differences between the mouse and human placenta.[142] In contrast, both rhesus CMV and guinea pig CMV are capable of crossing the placenta and causing congenital infection and are therefore useful models for studying the pathogenesis of congenital CMV infection.[401] Additional differences between the mouse and human placenta,[16] including placental development and timing of perfusion with maternal blood, can limit the utility of mice for the study of other viruses with known teratogenic effects (eg, ZIKV, HSV).

Key Concepts in Viral Pathogenesis

Virus-induced disease represents a complex series of interactions between the virus and the host. For any given virus, disease outcome can be influenced by a variety of virus-associated factors, such as viral genotype, viral variants that arise over the course of the infection, viral dose, and route of inoculation. Likewise, host factors, including genetic variation, underlying medical conditions (eg, diabetes), immune status, and

microbiome, as well as sex and age, affect susceptibility to disease or even the type of disease that develops. Given this complexity, it can be daunting to consider how to deconvolute these processes to understand specific mechanisms that contribute to viral pathogenesis. However, it is still possible to conceptualize virus-induced disease processes into discrete stages, mechanistically study the viral and host factors that regulate each of those stages, and even define aspects of those stages that are common between viruses. While the specific virus/host interactions that drive disease will be unique to any given virus, and even vary between individual hosts infected with the same virus, similar methodologies can be used to study the pathogenesis of different viruses and even define general principles that are common aspects of viral pathogenesis. There are a wide range of approaches for studying viral infection and disease, and we will discuss a few of these approaches to illustrate how these types of studies can inform our understanding of viral pathogenesis. Importantly, we will also discuss some of the limits of these approaches, while illustrating the importance of not overgeneralizing findings from one virus to others.

Clinical Observations Can Provide Fundamental Insights into Viral Pathogenesis

While much of our understanding of viral pathogenesis comes from experimental model systems, including animal models of virus-induced disease, fundamental insights are often provided by clinical observations of naturally infected individuals. For example, much of our understanding of measles virus–induced disease, including the incubation period between exposure and disease onset, the enhanced susceptibility of young individuals, and the concept of lifelong immunity following prior infection came from studies of a measles outbreak in the Fareo Islands in 1886[175,176] at a time when the viral cause of measles had not yet been identified. Likewise, during the modern era, the massive amount of clinical and epidemiological data generated by the SARS-CoV-2 pandemic has provided insight into the role of underlying heath conditions (eg, diabetes) and advanced age in disease susceptibility, while the success of anti-inflammatory treatments in reducing disease severity has illustrated a role of pulmonary inflammation as a driver of disease in some SARS-CoV-2 infected individuals.[38,107,252,396,468] Importantly, over the past 15 years the availability of more advanced analysis techniques for studying disease processes and host responses in patient populations, such as high throughput methods for quantifying genome wide gene expression (eg, proteomics and RNA sequencing), it is now possible to generate a more detailed picture of the molecular events occurring in an infected patient or tissue. However, as noted above, it is often difficult to fully dissect these disease processes at the mechanistic level through clinical analysis alone, and therefore, clinical findings often serve as a starting point for more detailed analysis using in *vitro* and *in vivo* model systems. Through the careful analysis of human clinical data and the use of appropriate cell culture based systems and animal models, it is often possible to define the molecular mechanisms underlying specific virus-induced disease processes.

Viral Pathogenesis as a Series of Sequential Stages in Infection

One approach for studying viral pathogenesis is to divide the virus-induced disease process into a series of stages, including initial infection, viral dissemination, invasion of the target tissue(s), and disease induction, followed by outcomes such as disease resolution/viral clearance, viral persistence/chronic disease, or even death of the host. By dividing these processes into discrete stages, it is often possible to understand how different viral and host variables affect each stage of the infection and disease process.

The pathogenesis of poliovirus represents a classic example of how viral pathogenesis can be broken down into several discrete, sequential stages. The poliovirus epidemics of the first half of the 20th century led to a concerted effort to understand the pathogenesis of poliovirus infection and disease. This analysis was key to understanding both the mechanisms of poliovirus transmission and why some individuals remained symptom free while others progressed to meningoencephalitis and paralysis. Importantly, this work also set the stage for the development of vaccines that have nearly eliminated poliovirus as a global disease threat.[325,391,393]

As illustrated by Figure 8.6, poliovirus infection consists of several distinct stages that help explain viral disease pathogenesis. Poliovirus transmission occurs via the fecal-oral route and enters Peyer patches from the intestine, where the virus replicates in lymphoid cells. The virus then spreads to secondary replication sites via the blood, where the virus replicates and seeds a second round of viremia that can lead to CNS infection in some individuals through either viral transit across the blood-brain barrier or infection of peripheral nerves that mediate viral entry into the CNS.[46,180,325,355,391,393,399] Upon entering the CNS, the virus replicates in and kills motor neurons, leading to paralysis. These studies also identified an important role for the immune system in determining whether an individual progresses to CNS infection and disease. The host immune response is initiated at the time of viral replication in lymphoid tissue, and the host antibody response is involved in clearing poliovirus from the circulation, which can limit the virus's ability to transit into the CNS.[325,399] Antibody has also been shown to mediate clearance of poliovirus and other viral pathogens from neurons.[280,325,340,491,493] Therefore, the kinetics and quality of the host immune response can have a major role in determining whether individuals remain asymptomatic, or develop neurologic disease.

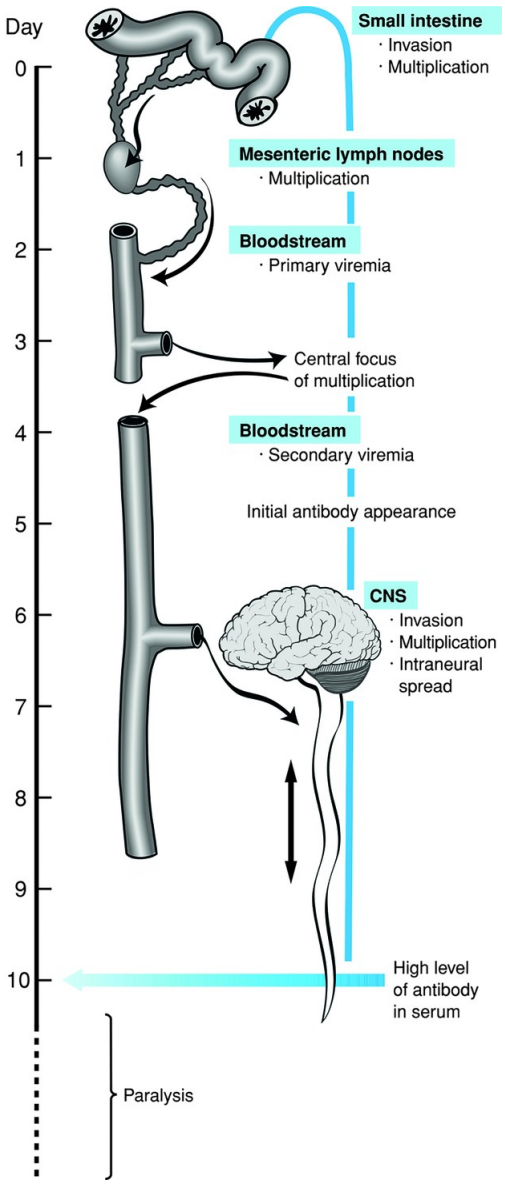

FIGURE 8.6 **Model of poliovirus pathogenesis as a series of sequential stages**. The steps in poliovirus infection of a human are schematized. (This figure was adapted from Chapter 22 in Fields Virology, Third Edition by Herbert W. Virgin IV.)

It is important to note that unrelated viruses that cause similar disease manifestations may go through a similar set of disease stages. For example, encephalitic viruses ranging from DNA viruses such as HSV, to RNA viruses, including polioviruses, rabies virus, WNV, or Venezuelan equine encephalitis virus (VEEV) must all invade the CNS to cause neurologic disease. While neuroinvasion is a common step in the disease process for all encephalitic viruses, the specific mechanisms or even routes of neuroinvasion vary greatly between viruses. HSV enters the CNS through retrograde neuronal transport via peripheral nerves such as olfactory nerves.[37,287,330] Rabies virus also enters the CNS via retrograde transport in peripheral nerves following virus uptake at neuromuscular junctions.[249] WNV can utilize olfactory nerves to enter the CNS[347]; however, the initial invasion of the CNS is mediated by disruption of the blood-brain barrier by inflammatory cytokines in the periphery.[18,517,520] VEEV initially enters the CNS via either olfactory nerves or the trigeminal ganglion[83]; however, a second round of neuroinvasion occurs via disruption of the blood-brain barrier; inhibition of blood-brain barrier disruption results in delayed onset of VEEV-induced disease.[425] As noted above, poliovirus can also enter the CNS either by directly crossing the blood-brain barrier or via infection of peripheral nerves.[46,180,325,355,391,393,399] Therefore, while viruses that cause similar disease states (eg, neurologic disease) may go through similar stages in the disease process, the specific molecular mechanisms used by each virus to complete each stage may be quite different. Recognizing how these virus-specific aspects of the disease process affect disease outcome is essential for understanding the pathogenesis of a given virus.

Mosquito-borne arboviruses, which include members of the order *Bunyavirales*, and the *Flaviviridae* and *Togaviridae* families, further illustrate the point that viral infection can be divided into discrete stages that share features across many viruses, while also demonstrating that viruses that cause very similar disease outcomes may do so through different mechanisms. These arboviruses all initiate infection via transcutaneous delivery in arthropod saliva, and while their initial target cells differ, initial viral infection and amplification occurs in resident cells in the skin.[41,289,304,469] Local viral replication seeds serum viremia, which facilitates transmission to new mosquito vectors if a sufficiently high viral load is achieved in the blood.[374,398,480] This viremia also facilitates viral dissemination to secondary sites of viral replication, including downstream targets of virus-induced disease. The disease states and target tissues associated with different arboviruses can also be quite similar. A wide variety of arboviruses from all three viral families cause neurologic disease, and as noted above, neuroinvasion therefore represents a key disease stage for each of these viruses. Likewise, unrelated viruses, such as YFV and Rift Valley fever virus (RVFV) can target

similar tissues (eg, the liver) and cause similar disease manifestations, such as hepatitis and hemorrhage.[213,328,389,390]

Comparing and contrasting the disease processes induced by phenotypically similar viruses can be useful for identifying common mediators of specific stages in pathogenesis, while also illuminating aspects of pathogenesis that are unique to a given viral pathogen. Advances in molecular virology, high throughput genetic screening, genomic technologies, and other experimental tools, as well as the use of animal models that reproduce key aspects of virus-induced disease, have allowed detailed analysis of the viral and host factors that regulate the various stages in the infection process for a wide variety of viruses at the molecular level. These approaches have identified cases where viruses that cause similar disease outcomes utilize common pathways. For example, arthritis-associated alphaviruses, including CHIKV, Ross River virus (RRV), and Mayaro virus (MAYV) all utilize Mxra8 as a cellular entry receptor,[544] while similar components of the host immune response, such as inflammatory monocytes/macrophages, contribute to the tissue pathology and rheumatic disease signs caused by these virus infections.[106,158,285,286,413] However, it is often the case that viruses that cause very similar disease outcomes interact with the host in very different ways to establish infection, avoid or suppress the host immune response, and ultimately cause disease. This is illustrated by VEEV and EEEV, mosquito-borne alphaviruses that cause severe neurologic disease. Although VEEV and EEEV must both establish infection after mosquito delivery, disseminate systemically, and invade the CNS to cause disease, the two viruses differ greatly in their interactions within the host at the molecular level. Following introduction into the skin via mosquito bite, VEEV infects Langerhans cells and/or dermal dendritic cells[304] through interactions with DC-SIGN,[247] which binds high mannose N-linked glycans on the surface of the mosquito-derived virus, and the viral entry receptor low-density lipoprotein receptor class A domain containing 3 (LDLRAD3).[42,302,304] The virally infected dendritic cells then migrate to the draining lymph node where VEEV replicates and spreads to secondary lymphoid tissues, while seeding a serum viremia that facilitates neuroinvasion via infection of olfactory nerves or the trigeminal ganglion.[83] In contrast, EEEV does not infect dendritic cells or other myeloid cells and instead initially infects skin fibroblasts and muscle cells. Unlike VEEV, EEEV does not utilize LDLRAD3 as an entry receptor[153] but instead uses VLDLR and ApoER2, two other lipoprotein receptors.[94] While receptor differences may influence VEEV and EEEV tropism, EEEV tropism is largely mediated by the presence of micro-RNA binding sites in the EEEV in the 3′ UTR that are targeted by miR-142-3P micro-RNAs expressed in myeloid cells.[485] Disruption of these miRNA target sites allows EEEV to replicate in myeloid cells, but these mutant viruses are attenuated and induce high levels of type I interferon, indicating that EEEV evades the host innate immune response by limiting viral replication in myeloid cells to promote viral dissemination and virus-induced disease.[157,248,485] EEEV and VEEV also differ significantly in other mechanisms related to the virus-induced disease process. The presence of positively charged mutations in the E2 glycoprotein of VEEV, as well as other alphaviruses such as CHIKV that arise through cell culture adaptation and promote binding to heparan sulfate and other charged glycosominoglycans, are associated with viral attenuation.[22,42,414] In contrast natural isolates of EEEV exhibit heparan sulfate binding activity, and this heparin binding activity contributes to EEEV neurovirulence in mouse models of EEEV-induced encephalitis.[154,155]

These examples illustrate several important points when thinking about viral pathogenesis. It is important to remember that even viruses from the same family that exhibit similar disease outcomes may do so through very different mechanisms. Understanding these mechanisms and how they differ between viruses has important implications both for understanding virus-induced disease processes and the development of antiviral therapies or vaccines. For example, blocking VEEV entry receptor LDLRAD3 has therapeutic benefit against VEEV in animal models,[302] since EEEV does not use LDLRAD3 as an entry receptor, this approach would be unlikely to have clinical benefit against EEEV. Likewise, introduction of heparin binding mutations is a common strategy for developing live attenuated vaccines against alphaviruses such as VEEV and CHIKV.[156] However, since heparin binding promotes EEEV pathogenesis,[154,155] this approach would not be beneficial in EEEV vaccine development.

Viral Genetic Determinants Can Impact Viral Pathogenesis at Multiple Levels

Viral genetic factors play important roles in the pathogenesis of all disease-associated viruses. These include viral genes and noncoding elements that regulate fundamental aspects of the viral lifecycle, including genome replication, receptor interactions, and virion assembly and that modulate the host response to viral infection. While many viruses are genetically stable, viral genetic adaptation plays an important role the pathogenesis of a wide variety of viruses. These adaptive mutations can affect the virus-induced disease at the population level, such as adaptive mutations affecting virus host range, or within individuals by driving immune evasion or selecting for viruses with enhanced tropism for specific organ systems. Understanding how these adaptive mutations affect virus-induced disease can provide important insights into the role of specific viral genes in the disease process, illuminate key interactions between the virus and the host, and even predict which viruses represent the greatest risk for zoonotic emergence.

Viral adaptation frequently occurs when viruses infect a new species and may even be a driver of zoonotic emergence. For example, both the original SARS-CoV and SARS-CoV-2 utilize angiotensin converting enzyme 2 (ACE2) as a cellular entry receptor.[217,263] Comparison of SARS-CoV isolates from early vs late stages in the 2003 outbreak identified adaptive changes in the receptor binding domain (RBD) of the viral spike protein that improved interactions with the human ACE2 receptor, thereby enhancing viral infectivity for human cells.[232,443] Similar adaptive changes have been observed during the SARS-CoV-2 pandemic, where viral variants that either enhanced virion stability[380] or improved ACE2 binding affinity resulted in enhanced transmissibility, increased viral replication efficiency in the lungs, and enhanced virulence. Moreover, these variants have emerged to become the dominant viral strain either regionally and/or globally.[327,417] Therefore, these types of adaptive changes can have major effects on viral pathogenesis and even enhance the virulence of newly emerging viruses as they adapt to a new host species.

While viral genetic changes can promote adaptation to a new host, and thereby enhance viral transmission or pathogenesis, there are also examples of viruses becoming less virulent as they adapt to new host species. This is illustrated by classic studies conducted by Fenner and colleagues on the pathogenesis and epidemiology of the myxomatosis virus in Australian rabbit populations.[136,137,139] European rabbits (*Oryctolagus cuniculus*) are an invasive species that causes significant ecological damage in Australia due to a lack of natural predators. Myxomatosis virus is a pox virus that causes mild disease in its natural host (*Sylvilagus brasiliensis*) but causes severe systemic disease with >99% mortality of infected European rabbits and was therefore introduced into the Australian rabbit population in 1950 as a biological control effort. The virus caused high mortality in rabbit populations at the time of its introduction, with initial mortality rates in excess of 99%. However, within a year mortality dropped to < 90%.[136,137,139] While there was clearly selection for resistance within the Australian rabbit population (Table 8.1), the virus also exhibited significant changes in virulence.[138,139] Myxomatosis virus isolates could be classified into five virulence grades based on their case mortality rate,[137] with grade 1 causing >99% mortality, while grade 3 causes 70%-90% mortality (Table 8.2). Although the virus introduced into the Australian Rabbit population was a Grade 1 virus, Grade 3 viruses quickly became the predominant circulating virus.[136,137,139] A second myxomatosis introduction in Europe in 1952 resulted in a very similar pattern, where the initial highly

virulent viruses were replaced by attenuated strains with reduced mortality rates and extended survival times.[137] Further studies also found that when a virulent strain was introduced into an area where viruses of intermediate virulence were circulating, the attenuated strains outcompeted the more virulent virus[136] as illustrated in Figure 8.7. However, there is also evidence that as rabbit populations became more resistant to myxomatosis virus, there was selection for increased viral virulence in some populations.[241] This led to the conclusion that the virus achieved equilibrium with its host, where highly virulent viruses may spread less efficiently due to the rapid death of the host, while overly attenuated viruses may not achieve sufficient viral loads for efficient transmission via direct contact or to arthropod vectors. Therefore, viruses of intermediate virulence would have a transmission advantage leading to these viruses outcompeting both highly virulent and highly attenuated viruses.[241]

TABLE 8.1 The susceptibility of nonimmune wild rabbits after successive epidemics of Myxomatosis

No. of Epidemics	Severity of Disease[a] (%)			
	Fatality Rate	Severe Disease Including Fatalities	Moderate Disease	Mild Disease
0	90	93	5	2
2	88	95	5	0
3	80	93	5	2
4	50	61	26	12
5	53	75	14	11
7	30	54	16	30

[a]Rabbits were challenged with a virus of grade III (intermediate) virulence. (See Table 8.2).

Adapted with permission of The Royal Society (U.K.) from Fenner FJ. The Florey lecture, 1983. Biological control, as exemplified by smallpox eradication and myxomatosis. *Proc R Soc Lond B Biol Sci*. 1983;218(1212):259-285; permission conveyed through Copyright Clearance Center, Inc.

TABLE 8.2 Virulence of field isolates of myxoma virus, 1951-1981

	Virulence Grade					
	I	II	III	IV	V	
Fatality rate (%)	>99	95-99	70-95	50-70	<50	
Mean survival time (d)	<13	14-16	17-28	29-70	NA	
Years	**% of isolates**					**# of samples**
1950-51	100	0.0	0.0	0.0	0.0	1
1952-55	13	20	53	13	0.0	60
1955-58	0.7	5	55	24	15	432
1959-63	1.7	11	61	22	5	449
1964-66	0.7	0.3	64	34	1.3	306
1967-69	0.0	0.0	62	36	1.7	229
1970-74	0.6	5	74	21	0.0	174
1975-81	1.9	3	67	28	0.0	212

NA, not applicable.

This table was adapted from table 15-2 of Nathanson N. *Viral Pathogenesis*. 1st ed. Raven; 1997, Ref.[343] and is based on data from Fenner F. The Florey lecture, 1983. Biological control, as exemplified by smallpox eradication and myxomatosis. *Proc R Soc Lond B Biol Sci*. 1983;218:259-285. doi:10.1098/rspb.1983.0039

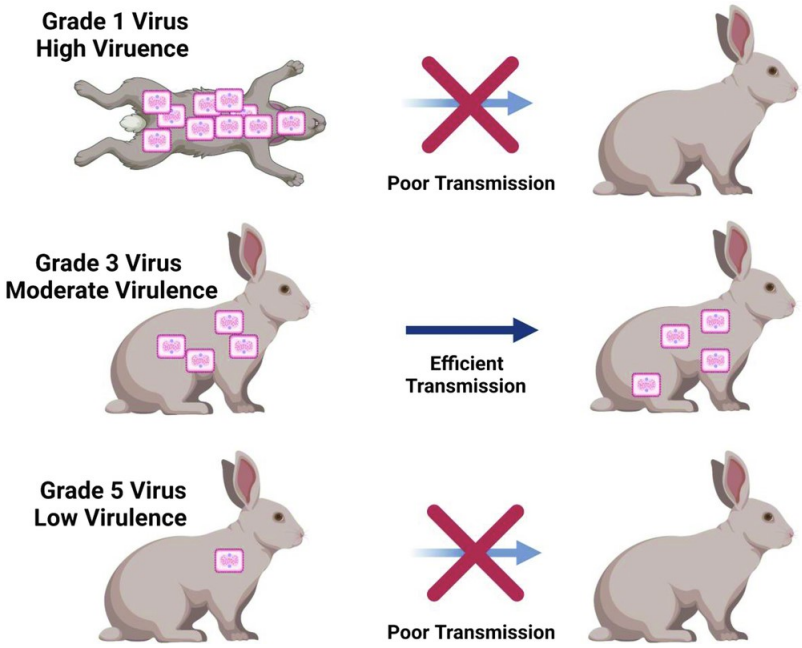

FIGURE 8.7 **Changes in viral virulence can affect transmission efficiency and lead to the establishment of equilibrium within a host population**. Highly virulent myxomavirus strains (Grade 1) result in the rapid death of the host, which limits the opportunity for viral transmission to new, susceptible hosts **(Top Panel)**. Likewise, viruses with significantly reduced virulence (Grade 5) produce less virus resulting in less efficient transmission **(Lower Panel)**. Viruses with intermediate virulence (Grade 3) result in sufficiently high levels of virus production, but delayed death in the host, which increases the likelihood that the virus will be transmitted to a new host **(Middle Panel)**. (Created with BioRender.com.)

The availability of high throughput, whole genome sequencing methodologies has allowed analysis of the genetic differences between the originally introduced Grade 1 myxomatosis viruses and the subsequent less virulent viruses that arose over the course of the outbreak.[241] These studies demonstrated that myxomatosis virus had undergone extensive evolution, and that less virulent viruses acquired mutations in a variety of virulence genes.[241] Importantly, the data also indicated that attenuation was not associated with specific mutations or even mutations in the same sets of genes between viruses, illustrating the stochastic nature of viral evolution during a major outbreak, and the possibility that different mutations or mutations in different genes may result in very similar pathogenesis phenotypes.

While coronaviruses and myxomatosis virus provide excellent examples of how viral genetic changes can impact virus-induced disease across populations, viral adaptive changes can also influence disease outcomes within individuals. This is illustrated by viral immune escape variants, where viruses avoid clearance by mutating T-cell and B-cell epitopes. Studies with LCMV in transgenic mice whose T cells were specific for a LCMV epitope in the viral glycoprotein demonstrated the rapid selection for viruses with mutations in this epitope that allowed for immune escape.[378] Similar results have been observed for other viruses. Lentiviruses such as HIV and SIV acquire mutations in $CD8^+$ T-cell epitopes, and these mutations are associated with escape from virus-specific T cells and progression to AIDS.[12,30,31,53,130,170,254,378] HIV also acquires resistance to neutralizing antibodies via adaptive mutations. Although individuals can mount neutralizing antibody responses against HIV, the neutralization-sensitive viruses are replaced by successive populations of neutralization-resistant viruses.[524] Importantly, unlike evasion of T-cell responses, which involves mutations that disrupt protective T-cell epitopes, HIV can escape virus-specific neutralizing antibodies through mutations that alter glycosylation sites on the viral envelope protein, where the glycans block antibody interactions with neutralizing epitopes.[524]

Conceptualizing Viral Pathogenesis as the Integrated Effects of Viral and Host Properties

Viral genetic factors clearly play a major role in determining viral virulence and can affect aspects of the disease process such as host range, tissue tropism, and immune evasion. However, variation in a wide variety of host factors also have a major impact on viral pathogenesis, and the interactions between these host factors and viral genetic determinants shape the virus-induced disease process (see Fig. 8.8).

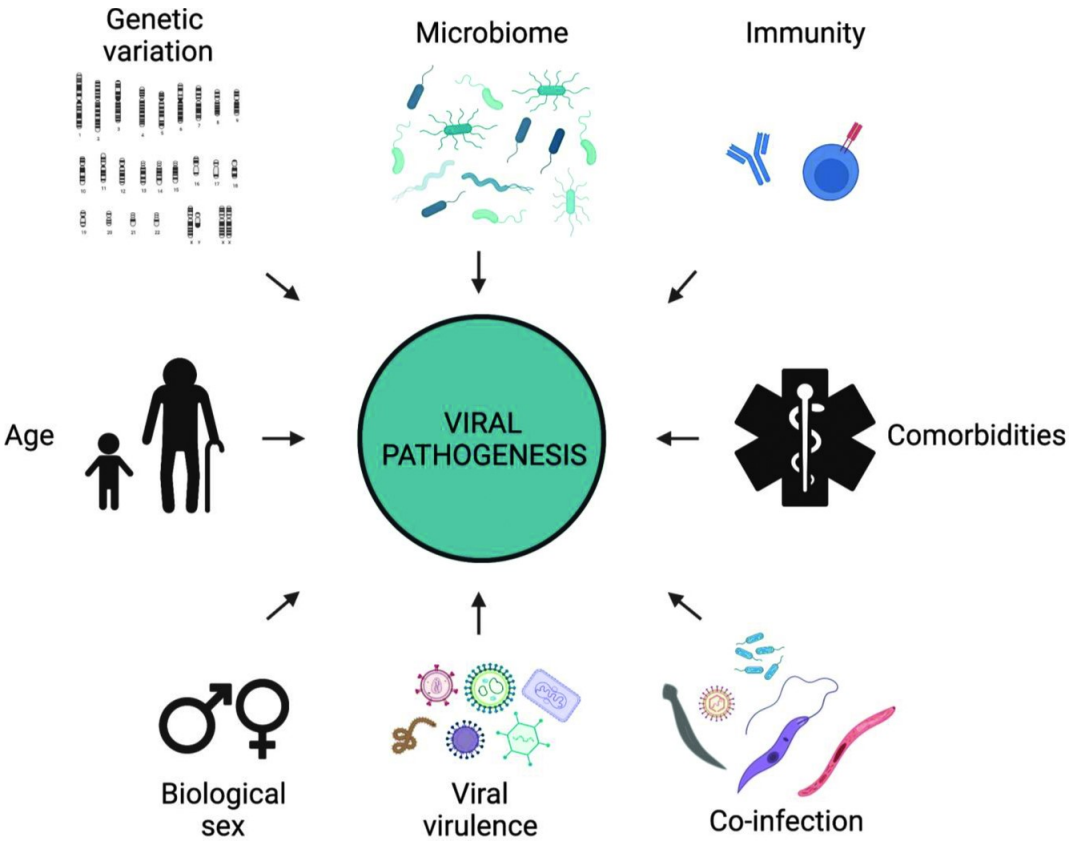

FIGURE 8.8 **Multiple viral and host factors interact to shape the pathogenesis of virus-induced disease**. In addition to virus-derived factors (eg, viral virulence, viral dose); host factors, including age, sex, genetic variation, microbiome composition, and underlying disease states (eg, comorbidities or coinfections); as well as host immune status (eg, preexisting immunity, immunodeficiency) can influence an individual's susceptibility to virus-induced disease. Different combinations of these host factors can contribute to variation in disease outcomes across a population infected by the same virus. (Created with BioRender.com.)

Effects of Host Genetic Variation on Susceptibility to Viral Infection

Major host determinants of viral virulence and pathogenesis are innate and adaptive immunity (discussed in more detail below), but host genes not involved immunity also play a role. Thus, allelic variations in host genes can alter viral pathogenesis. A powerful demonstration of this is the identification of inborn errors of immunity that are associated with severe outcomes of viral infection. For example, life-threatening encephalitis is a rare complication of herpes simplex virus-1 (HSV-1) infection.[379] Genetic studies of HSV-1 encephalitis patients revealed that this rare but severe outcome is associated with allelic variation that impairs type I and type III IFN-mediated immunity, including genetic mutations in STAT1,[126] a transcription factor activated downstream of the type I IFN receptor; NF-kappa B essential modulator (NEMO),[387] a protein that regulates the activation of IKKα and IKKβ; UNC-93B, a protein required for TLR3, TLR7, and TLR9 activation; TRIF[419] and TRAF3,[373,545] proteins required for TLR3 signaling; and TLR3,[545] a pattern recognition receptor that elicits induction of type I and III IFN. Loss-of-function variants in genes that regulate type I IFN immunity, including *IFNAR1*, *IRF7*, *IRF9*, *TLR3*, *TLR7*, and others, also have been associated with life-threatening COVID-19,[21,542] a rare complication following SARS-CoV-2 infection.

Among additional examples, some are well established while others come from single studies and await confirmation. Mutations in CCR5, which is a co-receptor for HIV entry,[11] confer resistance to HIV infection.[105,292,418] Human noroviruses (type virus Norwalk) are responsible for more than 90% of the epidemic nonbacterial gastroenteritis in the world.[119,167] Norwalk virus susceptibility is determined by blood group secretor status conferred by the presence of the FUT2 fucosyltransferase.[208,292] Among human norovirus strains there are multiple patterns of norovirus viruslike particle (VLP) binding to blood group carbohydrates suggesting that allelic variation in human blood groups contribute to susceptibility to a variety of norovirus strains. Patients with mutations in the IFN-γ receptor have been reported to have unusual viral syndromes.[376] Autosomal dominant mutations in the chemokine receptor CXCR4 have been associated with severe warts,[273] and mutations in *EVR1* and *EVR2* have been associated with an unusual clinical presentation of papillomavirus infection called epidermodysplasia verruciformis.[394] Allelic variations in mannose binding lectin (MBL) and FcγRIIA have been linked to the severity of SARS-CoV-1.[216,539] A relationship between expression of certain KIR genes, encoding NK cell receptors, and severity and chronicity of infection with HIV, HCV, and EBV have been reported.[74] Responsiveness to hepatitis B vaccine may be linked to allelic variations in complement genes.[48,201] Thus, a significant number of allelic variations in humans are important determinants of viral pathogenesis.

Though many studies have focused on the impact of variation in a single gene on viral pathogenesis, it is clear that susceptibility or resistance to viral infections is a complex phenotype regulated by multiple interacting genes and gene networks. For example, studies designed to identify genes that regulate resistance to influenza A virus infection between two mouse strains identified multiple quantitative trait loci (QTL) that contribute to disease susceptibility.[51] Though the full number of host genes that vary between susceptible and resistant hosts for a single virus is incompletely understood, it is likely that variation in hundreds of different genes may contribute to disease resistance or susceptibility.[75,511] Advances in the area of host genetics, including the application of Genome Wide Association Studies (GWAS), which scan the genome for allelic variants, such as single nucleotide polymorphisms (SNPs), associated with specific phenotypes, as well as whole exome and whole genome

sequencing, promise to further enhance our understanding of how different polymorphic genes contribute to disease susceptibility. For example, application of GWAS approaches to identify genes that associate with HIV control identified a major association between polymorphisms in the HLA locus that affect class I peptide presentation to CD8 T cells and an individual's ability to control HIV.[214] These approaches have also been extended to HCV, and identified a polymorphism within the IL28B gene (interferon λIII) that associates with viral clearance following interferon therapy.[161] These types of GWAS studies have proven useful for identifying susceptibility loci for some viral pathogens and hold promise for predicting treatment outcomes for infection in patient populations as has been suggested for inflammatory diseases.[509]

The examples outlined above illustrate that allelic variants in host genes can have a significant impact on the pathogenesis of infection. As multiple independently segregating genes contribute to the susceptibility or resistance to a viral pathogen, many human genotypes exist that may vary significantly in their resistance to even a single viral infection. Therefore, even without considering the impact of viral virulence determinants or other environmental factors, much of the variation in viral pathogenesis within a diverse population could be explained by polymorphisms in host genes. This has implications not only for viral pathogenesis in human populations but also for studying viral pathogenesis in animal models. Though GWAS and candidate gene studies in humans can establish an association between a polymorphic gene and susceptibility to a viral infection, the investigation of the mechanisms by which a polymorphic gene or genes affect pathogenesis requires additional tools, including appropriate animal models (As Described Above in the Section on Animal Models).

Age as a Determinant of Viral Infection Outcomes

The age of the host can have a major impact on susceptibility to virus-induced disease, with both very young and very old individuals often being at increased risk of developing severe disease. Very young individuals often have increased viral tissue burdens, higher levels of viral dissemination, and increased susceptibility to viral invasion of target tissues, which correlates with disease severity or outcome.[448] For example, neonatal exposure to viruses such as HSV or CHIKV frequently results in severe neurologic disease that is less frequently observed in adults.[32,165] Furthermore, while the majority of adults clear HBV infection, HBV exposure in neonates commonly results in chronic infection.[528] These differences in susceptibility may be partly due to infants being immunologically naïve, and the lack of preexisting adaptive immunity leads to increases disease susceptibility. There is also evidence that the innate immune response is less robust in the very young compared to adults, and these differences in innate immunity lead to increased risk of developing severe disease for viruses such as HSV.[165] However, other developmental changes can also contribute to the enhanced susceptibility of the very young to infection. For example, studies with Sindbis virus, which shows age-dependent virulence in mouse models, have demonstrated that undifferentiated neurons are susceptible to virus-induced apoptosis, while differentiated neurons are resistant.[281]

Age dependence of virus resistance does not always favor older hosts. For example WNV encephalitis is more common and more severe in older hosts,[193] and lethality after SARS-CoV and SARS-CoV-2 infection is highly age dependent, with risk of severe disease being significantly higher in people older than 60 years of age compared to younger people.[120,181,371] This is also the case in mouse models of SARS-CoV and SARS-CoV-2 infection.[277,403,404] Alterations in the immune response in older individuals at least partly explain the increased susceptibility of aged individuals to severe virus-induced disease. Age-dependent immune senescence in older individuals can lead to waning of preexisting immunity, poor ability to mount adaptive immune responses,[67,100] as well as defects in key innate immune pathways, such as the type I IFN system, where dampened innate immune responses or even autoantibodies against type I IFN (eg, autoantibodies against type IFN account for up to 20% of fatal COVID-19 cases) can impact host antiviral responses.[35,135] Although the very young and the very old are often at increased risk of viral infection compared to healthy young adults, there are also instances where young adults are at increased risk of enhanced disease. For example, mumps virus is generally associated with painful swelling in the parotid glands (parotitis) but can cause swelling in the testis (orchitis) that can affect male fertility. Orchitis is considered to be rare in prepubescent males, but more than 30% of infections in postpubescent males can result in orchitis.[36] During the 1918 H1N1 influenza A virus pandemic, very young and old individuals exhibited increased death rates normally associated with influenza A virus infection. However, young adults in the 15- to 40-year age range also had more severe disease signs than did older children and teenagers.[366] While the mechanisms responsible for the increased susceptibility of young adults to 1918 influenza A virus are not fully understood, younger individuals had not previously been exposed to H1N1 influenza strains, and this lack of prior immunity would have left them susceptible to infection, while their more robust inflammatory responses may have resulted in so-called cytokine storms that contributed to pathogenesis.[108,235,366]

Biological Sex as a Determinant of Viral Infection Outcomes

Sex differences can influence the outcome of viral infections, and this has been highlighted by the COVID-19 pandemic. Severe COVID-19 is more commonly observed in males compared with females.[85,122,181,437] This male bias in severe disease also has been observed for the other highly pathogenic coronaviruses, SARS-CoV-1[279] and MERS-CoV.[10] The precise reason(s) for these observations is not well understood but could reflect sex-specific differences in steroids, chromosomes, and immune responses.[437] For example, male COVID-19 patients were found to have higher levels of cytokines such as IL-8 and IL-18, higher activation of monocytes, and less activation of T cells, which correlated with disease severity.[474] Studies in mice also demonstrated sex-based differences during CoV infection. Male mice are more susceptible to SARS-CoV-1 infection, which is characterized by elevated lung viral burden and enhanced lung inflammation and injury compared with age-matched female mice.[81] The less severe infection outcomes in female mice were linked with sex steroids as treatment of female mice with an estrogen receptor antagonist increased virus-induced mortality.[81] Similar findings were observed in SARS-CoV-2-infected hamsters in which male animals developed more severe lung pathology.[112] Sex-based differences in infection outcomes also have been observed for a variety of other viral infections, including WNV,[222] CHIKV,[44,65,220,503] and influenza A virus.[503]

Effects of Variation in the Host Microbiome on Susceptibility to Viral Infection

The host microbiota regulates immunity and thus functions as an additional host property that influences susceptibility to viral infection. Mice treated with oral antibiotics display differential outcomes to a variety of viral infections, including influenza A virus,[212,466] HSV-2,[353] CHIKV,[466] poliovirus,[268] reovirus,[268] norovirus,[28,224] rotavirus,[494] and numerous others. In the case of enteric viruses including poliovirus, reovirus, and rotavirus, antibiotic-treated mice are less susceptible to viral infection and virus-induced disease, indicating that in these cases the host microbiota promotes viral replication and pathogenesis. Mechanistically, poliovirus and reovirus infectivity is enhanced in the presence of intestinal bacteria or bacterial products.[40,268] Antibiotic treatment of mice also reduces replication of murine norovirus in the intestine during

acute infection[224] and prevents persistent enteric murine norovirus infection.[28] Additional studies revealed complex interactions between norovirus and the host microbiota. During acute murine norovirus infection, the intestinal microbiota limits viral replication in the proximal small intestine but promotes viral replication in more distal regions of the small intestine and the colon.[174] The inhibition of viral replication in the proximal small intestine was found to be due to microbiota-mediated transformation of bile acids (ie, deconjugated), which promote induction of type III IFN to locally restrict norovirus replication.[174] Intestinal bacteria–generated bile acids also can influence infection outcomes with nonenteric viruses. Depletion of the microbiome increases dissemination and tissue burdens of CHIKV due to impaired type I IFN induction in plasmacytoid dendritic cells (pDC).[531] Control of CHIKV infection and normal pDC type I IFN responses were restored in antibiotic-treated mice reconstituted with *Clostridium scindens* or treated with the secondary bile acid deoxycholic acid, which can be generated by *Clostridium scindens*. Although the mechanism(s) is not known, the gut microbiota also can regulate type I IFN responses in lung epithelial cells to limit influenza A virus infection.[58] These findings highlight how microbiota-generated metabolites can regulate immunity to viral infection, including at distal sites, and function as a determinant of viral pathogenesis.

Coinfection as a Determinant of Viral Infection Outcomes

An additional host property that can determine the susceptibility to virus infection is the presence of coinfection. For example, coinfection with *Mycobacterium tuberculosis* (TB) can accelerate the progression of disease during HIV-1 infection by a number of proposed mechanisms including T-cell exhaustion and TB-activated inflammation.[269,535] Enteric helminths commonly infect humans, and these infections can cause profound alterations in host metabolism and immunity that influence the outcomes of viral infection.[109] Prior infection of mice with the enteric helminth *Trichinella spiralis* diminished the development of antiviral immunity and enhanced the intestinal viral burden following murine norovirus infection.[364] These effects were independent of the host microbiota and were instead mediated by IL-4- and STAT6-dependent signaling stimulated by helminth infection. HSV-2 infection in the murine female genital tract also is more severe during helminth coinfection, likely due to helminth-induced recruitment of eosinophils and induction of a type 2 immune profile in the female genital tract.[86] Enteric helminth infection also can influence systemic viral infections as coinfection of mice with the intestinal roundworm *Heligmosomoides polygyrus bakeri* increased disease severity following WNV infection again by an IL-4 and STAT6-dependent mechanism.[110] In contrast, induction of IL-4 and its effects on $CD8^+$ T cells during infection with the flatworm *Schistosoma mansoni* or the intestinal roundworm *Nippostrongylus brasiliensis* enhanced control of a subsequent respiratory infection with murine γHV68,[407] and helminth coinfection has been shown to protect against other respiratory virus infections including influenza A virus.[428] Coinfection with protozoan parasites also can dictate susceptibility to virus infection. In clinical studies conducted during the Ebola outbreak in West Africa, Ebola virus–infected patients who had *Plasmodium* parasitemia were found to be more likely to survive.[411] In a mouse model, prior exposure of mice to *Plasmodium yoelii* protected from a subsequent infection with mouse-adapted Ebola virus.[406] These protective effects were mediated by *Plasmodium*-mediated induction of IFN-γ. Similarly, prior exposure of mice to *Plasmodium* species suppresses CHIKV replication and joint disease by the induction of IFN-γ.[478] These examples and many others demonstrate that coinfections can profoundly impact viral pathogenesis.

Effects of Comorbidities on Susceptibility to Viral Infection

Similar to coinfection, the presence of underlying noninfectious diseases and disorders at the time of virus exposure can impact the pathogenesis of viral infection. Perhaps the most common condition linked with altered viral pathogenesis is immunosuppression. For example, immunocompromised hosts, including induced immunosuppression for transplant recipients, autoimmunity, and other reasons, are highly susceptible to severe HCMV infection that can be characterized by pathology in a variety of tissues (eg, retinitis, pneumonitis, and hepatitis) not typically observed during HCMV infection of immunocompetent individuals.[179] As mentioned above, the COVID-19 pandemic has revealed that individuals with neutralizing autoantibodies against type I IFNs, another form of immunosuppression, are more susceptible to life-threatening SARS-CoV-2 infection.[34,35,297]

Obesity was identified as a risk factor for severe influenza during the 2009 influenza A H1N1 pandemic.[298,487,536] In animal studies, genetic and diet-induced obese mice develop more severe disease following infection with mouse-adapted influenza A virus and 2009 H1N1 influenza A virus.[350,458] The worse disease was characterized by differences in the pulmonary inflammatory response (ie, composition of lung infiltrates); more severe injury in lung tissue, including protein exudates and edema; and poor reconstitution of damaged airway epithelium. Similarly, mice with diet-induced obesity and type 2 diabetes are more susceptible to severe MERS-CoV infection characterized by poor wound healing and delayed recovery from virus-induced lung damage.[264] The impact of obesity and metabolic diseases on respiratory virus infection has been further highlighted during the COVID-19 pandemic; obesity, diabetes, and hyperglycemia are associated with an increased risk of severe SARS-CoV-2 infection.[133,375,385,451,467,468,518,537] Collectively, these limited examples illustrate that preexisting comorbidities are important host properties that determine viral pathogenesis.

Viral Interactions With the Host Immune System Have a Major Impact on Viral Pathogenesis

The host immune system, which can be broadly divided into innate and adaptive (eg, antigen specific) immune responses, has a major impact on the pathogenesis of most viral infections. At a fundamental level, the innate immune system initially limits viral replication, while adaptive immunity mediates viral control and clearance. Therefore, poor innate and/or adaptive immune responses may lead to adverse outcomes such as uncontrolled viral replication and severe disease or failure to clear infection and chronic disease. However, while robust immune responses are needed for viral control and clearance, an overactive or dysregulated immune response can lead to immune pathology characterized by excess tissue damage and disease (Fig. 8.9). Therefore, understanding how a virus interacts with the immune system can provide important insights into the pathogenesis of virus-induced disease. Given the complexity of the host immune system and its regulation, a comprehensive discussion of the host immune system, or even specific aspects of the innate or adaptive immune system, is beyond the scope of this chapter. We will therefore provide some key examples to illustrate the importance of virus/immune system interactions in viral pathogenesis. For readers who are interested in a more in-depth discussion of the host immune system and its role in the response to viral infection, please consider the following resources.[1,339]

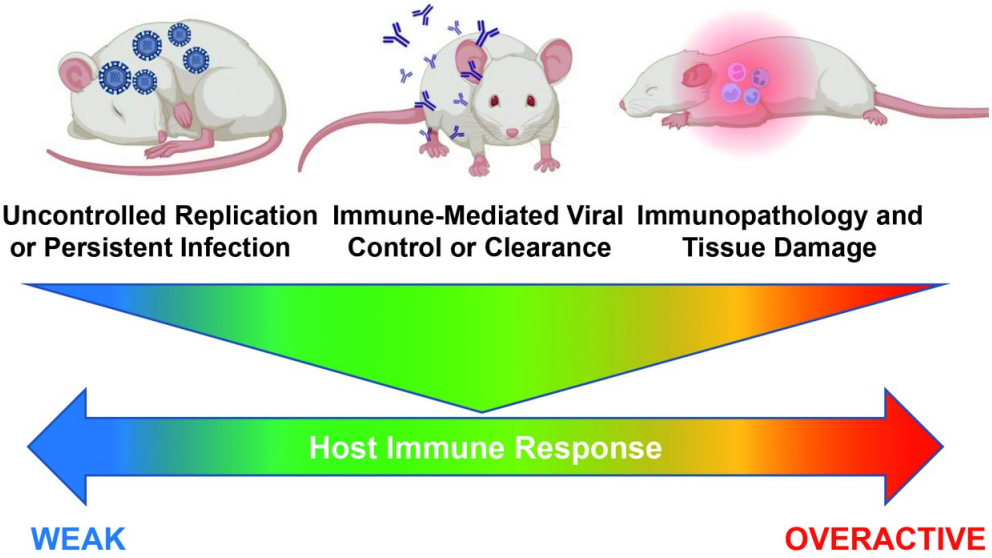

FIGURE 8.9 **Host immune responses mediate control of virus infection and immunopathology**. The strength of the host immune response can impact viral infection outcome. Individuals who mount weak or suboptimal immune responses may suffer from uncontrolled viral infection or persistence. Likewise, individuals who mount overactive or dysregulated immune responses can develop excess tissue damage and immune pathology. (Created with BioRender.com.)

The Host Immune System Plays an Essential Role in Controlling Viral Infection

The mammalian innate antiviral immune response consists of a variety of cell intrinsic antiviral restriction factors (eg, APOBEC3G), cytokines (eg, type I interferons), cell extrinsic defense pathways (eg, the complement cascade), and effector cells (ie, natural killer cells, neutrophils, and monocytes) that protect from viral infection through direct inhibition of the virus's ability to infect or replicate in cells, the induction of other antiviral molecules (eg, interferon stimulated genes of ISGs), or the killing of virally infected cells. The importance of these innate immune pathways for specific viruses has been demonstrated both in humans and in animal models. As discussed above, people with inborn genetic errors that result in defects in the induction of type I interferon responses, such as IRF7, exhibit enhanced susceptibility to a variety of viruses, including SARS-CoV-2 and Influenza A virus.[92,542] Likewise, NK cells play an important role in controlling herpesviruses, as demonstrated in mice infected with MCMV,[326] while humans suffering from NK cell deficiency or a loss of NK cell effector function are predisposed to recurrent herpesvirus infections.[361] In addition to directly controlling viral infections, innate immunity also influences the adaptive immune system by recruiting immune cells, promoting efficient antigen presentation and co-stimulation, and shaping the nature of antiviral antibody and T-cell responses. Therefore, the quality of the innate immune response to a virus can influence the efficacy of the adaptive immune response and thereby determine whether a viral infection is cleared or results in persistent infection and chronic disease.[551]

The innate immune system is composed of multiple effector molecules, signaling pathways, and cell types that play important roles in the host response to viral infection.[551] For example, the type I interferon system, as well as the related type II (IFNγ) and type III (IFNλ) interferons, plays a particularly important role in the response to viral infection. Interferons (IFN), which can act in both an autocrine and a paracrine manner, stimulate a wide array of IFN-stimulated genes (ISGs), which exhibit antiviral and immune modulatory activity.[432,434,495] As noted above, humans with genetic defects in key genes that regulate interferon induction or signaling are at significantly increased risk of developing severe virus-induced disease.[92,542] Likewise, studies in animal models have demonstrated that type I IFN signaling is essential for controlling a wide variety of viruses[533]; can regulate the tissue tropism or even host range of viruses such as Sindbis virus, myxoma virus, and ZIKV[415,516,533]; and also modulates the host adaptive immune response to influence viral clearance and protection from persistent infection.[551] The importance of the type I IFN system in controlling viral infections is further illustrated by the fact that most, if not all pathogenic human viruses have evolved strategies for evading detection by the host IFN system or actively antagonize key components of the IFN system to block type I IFN induction, IFN receptor signaling, or the activity of specific IFN-induced antiviral effector molecules. As discussed above, these virally encoded IFN antagonists are often essential for viral virulence. For example, the NSs protein of RVFV antagonizes the host type I IFN response by interfering with host transcription.[45,275] RVFV strains carrying mutations that disrupt NSs function are attenuated in wild-type animals, but these viruses regain full virulence in mice lacking a functional type I IFN system, demonstrating the importance of NSs-mediated IFN antagonism for the virus-induced disease process.[54]

The adaptive immune system also plays an essential role in the pathogenesis of most mammalian viruses. Although a comprehensive review of the adaptive immune system is beyond the scope of this chapter, virus-specific T-cell and antibody responses are essential for viral clearance, contribute to the control of persistent and/or latent viral infections, and protect from reinfection through the maintenance of long-term immune memory. Individuals with inborn immune deficiencies that affect T-cell function, HIV-induced acquired immunodeficiency syndrome (AIDS), or immune-suppressed individuals exhibit increased susceptibility to virus infections. For example, functional T-cell responses are essential for control of pox viruses, where vaccinia virus infection results in little or no disease in immunocompetent individuals, but T cell –deficient individuals are at risk of progressive vaccinia that can be fatal.[506] These individuals are also susceptible to certain virus-induced cancers, such as Kaposi sarcoma; suffer from recurrent herpesvirus infections; and exhibit defects in clearance of viruses such as noroviruses and SARS-CoV-2.[412] Although T cell–mediated immunity is generally thought to be more important for control and clearance of viral infections, and persons suffering from B-cell deficiencies or agammaglobulinemia (antibody deficiency) are at lower risk of severe viral infection than those suffering from T-cell deficiency, humoral immunity plays roles in the control of infections and is often the main correlate of immune protection from reinfection following either natural infection or vaccination.[173] B-cell or antibody deficiency, as well as treatment with B cell–depleting therapies, has been associated with persistent infection or severe disease outcomes for viruses such as WNV, human norovirus, and SARS-CoV-2.[88,202,251,365,479]

Heterologous Immunity

Heterologous immunity, or immunity to prior infections that impacts the outcome of subsequent infection with a distinct infectious agent, can have profound effects on viral pathogenesis that range from complete protection to severe immunopathology and disease enhancement. Heterologous immunity is typically mediated by cross-reactive T cell or antibodies, but other effectors of immunity also can contribute. Mechanisms of heterologous immunity include direct cross-reactivity between unrelated viruses, nonspecific activation of bystander cells, cytokine-mediated activation of cells that engage residual antigen from prior infections, and virus infection mediated induction (eg, via cytokines or cell injury) of self-antigen. All of these mechanisms can alter T and B cell memory responses, repertoires, and immunodominance leading to variation in the outcome of viral infection.[525]

A prominent example of heterologous immunity impacting viral pathogenesis is observed with DENV infection (Fig. 8.10). A major risk factor for clinically severe dengue (ie, dengue hemorrhagic fever/dengue shock syndrome) is prior infection with a distinct DENV serotype.[93,420,529] In addition, DENV infection in infants born to dengue-immune mothers when maternal antibodies wane to subneutralizing levels also is associated with increased risk of severe dengue.[84,246,450] A number of studies suggest that the immunological mechanism for these observations is antibody-mediated enhancement (ADE) of DENV infection and disease[190,238] in which heterotypic antibodies, at specific concentrations, bind but do not neutralize DENV virions during the secondary infection. These antibody-coated virions are recognized by Fcγ receptors that mediate virus internalization and thus enhance DENV replication in leukocytes. This idea is further supported by studies showing that patients with DENV-specific afucosylated IgG1 with enhanced affinity for FcγRIIIA had more severe disease.[55,521]

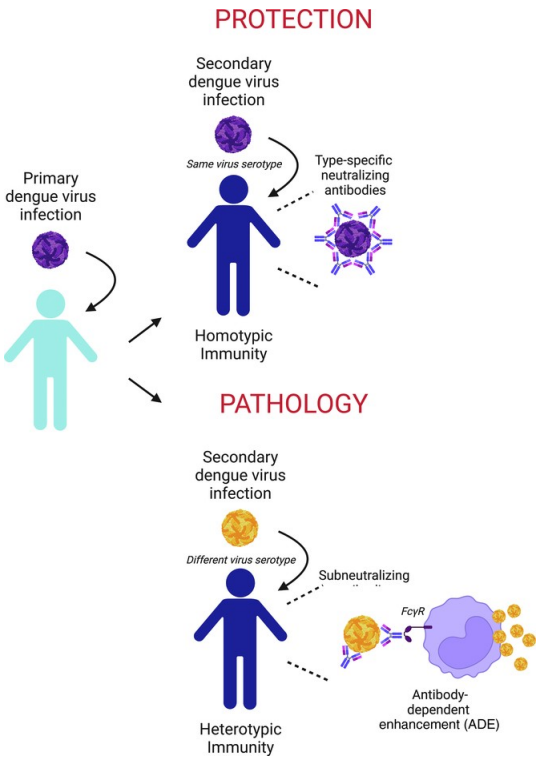

FIGURE 8.10 **Heterologous immunity affects outcomes of dengue virus infection**. The outcome of a secondary infection with dengue virus is dictated by the preexistence of homologous or heterologous immunity, with the latter associated with protection and the former associated with pathology due to the enhancement of dengue virus infection and disease by cross-reactive subneutralizing antibodies (nonneutralizing antibodies that bind the virion or neutralizing antibodies below the threshold concentration for neutralization). (Created with BioRender.com.)

During the SARS-CoV-2 pandemic, an important question has been whether prior infection with a common seasonal human CoV (ie, HKU1, OC43, 229E, and NL63) alters the outcome of SARS-CoV-2 infection. Indeed, some clinical studies found that patients with a previous seasonal human CoV infection developed less severe COVID-19, although there was no difference in the rate of SARS-CoV-2 infection.[55a] These data suggest that preexisting immunity against a seasonal hCoV can protect against severe SARS-CoV-2 infection. Additional studies found that some individuals possess nonneutralizing antibodies that cross-react with SARS-CoV-2 spike and nucleocapsid proteins[17]; however, these antibodies were not associated with protection against SARS-CoV-2 infection or severe COVID-19, suggesting that other immunological mechanisms such as cellular immunity may mediate heterologous protective immunity.

These are just two examples from a variety of studies indicating that heterologous immunity influences resistance and susceptibility to viral infection and disease.[441,525]

Viral Clearance and Chronic Viral Infection

The host immune system plays an essential role in the clearance of viral infections. These interactions can be roughly broken down into four categories: (1) clearance of acute infection with lifelong sterilizing immunity, (2) clearance of acute infection with transient immunity and reinfection, (3) viral latency, and (4) viral persistence with active viral replication. The immune system plays a fundamental role in each of these viral infection outcomes. Many viruses, such as YFV, Venezuelan equine encephalitis, and variola virus, the cause of smallpox, are associated with acute infection, and provided the host survives the infection, the immune system rapidly clears the virus and confers lifelong immunity to

reinfection. Other viruses, such as respiratory syncytial virus (RSV), noroviruses, and seasonal coronaviruses also cause acute infection and are cleared by the immune response. However, antiviral immunity against these viruses is often transient, allowing viral reinfection to occur as immunity wanes.[131] For example, RSV is a common respiratory infection in children that can cause serious respiratory disease, especially in preterm infants or young children with underlying medical conditions.[497] While the host immune system clears acute RSV infection, adaptive immune responses to the initial infection are weak, allowing reinfection to occur. Although subsequent infections result in more robust immunity and reduced disease susceptibility, RSV can continue to reinfect individuals into adulthood and is a common cause of severe respiratory infection in the elderly as antiviral immunity wanes.[497]

Many viruses are associated with chronic disease in the form of either viral latency or persistent infection with ongoing viral replication, and the host immune response plays an important role in regulating these types of infections. Herpesviruses have both lytic and latent phases in their replication cycle, and following the establishment of viral latency, herpesvirus can undergo frequent reactivation events leading to lytic replication and virus production. The host immune system plays an important role in regulating both lytic and latent stages of the herpesvirus life cycle. For example, NK cells and cytotoxic T cells are essential for controlling acute herpesvirus infection, such as MCMV, and individuals with defects in NK-cell or T-cell function are at increased risk of recurrent herpesvirus infection.[13,102,195,300,361,397] Importantly, these viruses have evolved a wide variety of mechanisms for modulating both NK-cell and T-cell recognition to allow the virus to avoid immune clearance during acute infection, while viral latency limits or eliminates production of viral proteins, allowing herpesviruses to avoid immune detection during latency.[361] Other viruses, such as HIV and HCV, cause persistent infection characterized by continuous viral replication. Although the host immune response controls the initial burst of viral replication, the immune response is unable to clear infection due to the viruses' high mutation rate, which results in the rapid generation of viral escape variants that are able to evade antiviral T cells or antibody-mediated neutralization and maintain viral persistence.[70,223] The failure of the immune system to clear these types of persistent infections can have significant consequences for the host, including organ failure or cancer. For example chronic HBV infection is associated with increased rates of cirrhosis, can lead to liver failure, and significantly increases the risk of developing hepatocellular carcinoma.[183]

Immune Clearance of Viral Infection Can Lead to Tissue Damage and Disease

The host immune system employs both cytolytic and noncytolytic strategies for clearing viruses from infected cells and tissues. Noncytolytic mechanisms, which preserve the virally infected cell while clearing the virus, are important for minimizing tissue damage or preserving nonrenewable cell types such as neurons. Virus-specific antibodies play an important role in the clearance of Sindbis virus,[280] and nonneutralizing antibodies that bind to the viral E2 glycoprotein on the surface of infected neurons act in combination with IFN-γ to suppress viral replication without killing the cell.[177,280] Antibodies can also enter infected cells to inhibit replication of viruses such as reovirus and WNV without killing the infected cell,[483,507,508] and the tripart motif containing-21 (TRIM21) protein acts as an intracellular antibody receptor that can bind viruses opsonized with nonneutralizing antibody and promote viral degradation while inducing the activation of antiviral pathways.[143,305]

The immune system also employs a number of cell extrinsic mechanisms to initiate cell killing via apoptosis or other cell death pathways. These effectors include cytokines such as TNF and the complement cascade. NK cells and cytotoxic T cells also kill virally infected cells via cytotoxic granule components that are introduced into virally infected cells via perforin, and which have been shown to be important for the control of a variety of viruses, including herpesviruses, coronaviruses, arenaviruses, picornaviruses, and flaviviruses.[236,295,296,337,372,446]

Certain viruses, including HBV and LCMV, can persistently replicate without killing the infected cell. Therefore, viruses such as HBV can infect cells and replicate within tissues without causing significant cell death, tissue destruction, or disease for several weeks. However, liver damage and disease, as defined by release of serum alanine aminotransferase (sALT), a marker of hepatocyte lysis, occurs at later stages in the infection process in concert with the appearance of CD8 T cells and the expression of IFN-γ in the liver (Fig. 8.11).[527,528] Studies using HBV transgenic mice have demonstrated that these cytolytic CD8 T cells lyse infected cells via perforin and FAS dependent cell killing pathways. These CD8 T cells are essential for viral clearance but also contribute to acute liver damage through both direct cell killing and the recruitment of other inflammatory cell populations into the liver.[141,482] Therefore, while the immune response plays a key role in clearing virus from the liver, inflammatory damage and acute hepatitis are a consequence of this protective immune response.

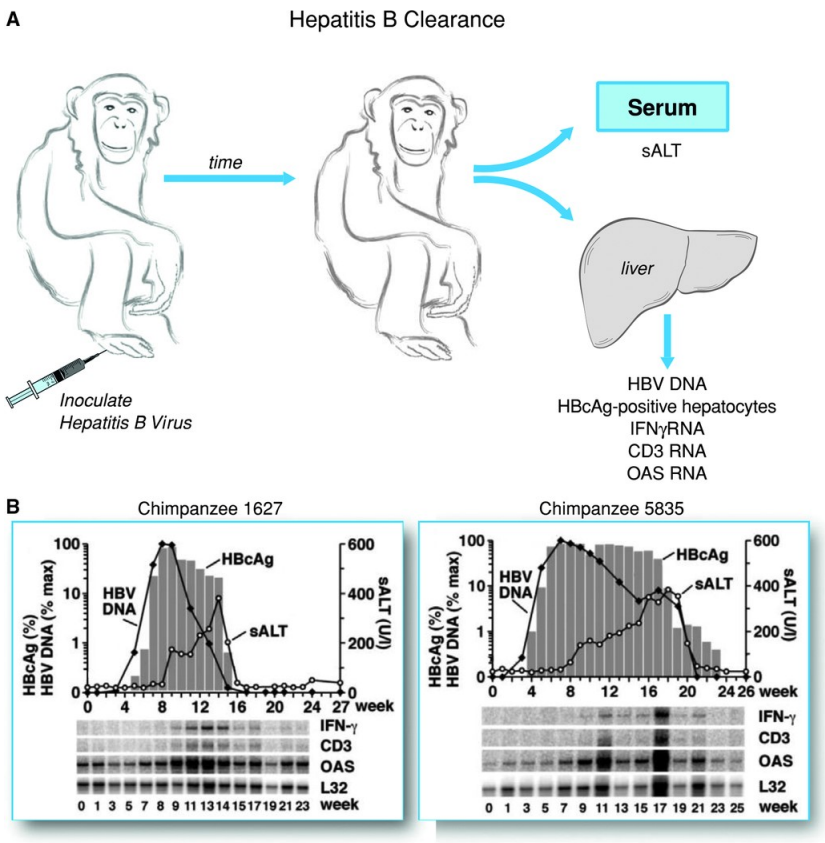

FIGURE 8.11 **Clearance of hepatitis B virus is associated with liver damage**. Chimpanzees were infected with HBV as illustrated in **panel A**, and at various times after infection serum samples or liver biopsies were taken and analyzed for serum alanine aminotransferase (sALT) and various parameters of HBV infection **panel B**. Intrahepatic HBV DNA (*black squares*, Panel B) is expressed as a percentage (% max) of the peak HBV DNA levels in the liver of each animal. Hepatitis B core antigen-positive (HbcAg) hepatocytes (*gray bars*) are expressed as percentage of the total number of hepatocytes. sALT (*open circles*) is expressed in units per liter. Intrahepatic expression profiles for IFN-γ, CD3, and 2′5′-OAS were determined by RNase protection assay. (Adapted by H.W. Virgin IV from Wieland S, Thimme R, Purcell RH, et al. Genomic analysis of the host response to hepatitis B virus infection. *Proc Natl Acad Sci U S A*. 2004;101(17):6669-6674. Copyright © 2004 National Academy of Sciences, USA.)

Overactive or Dysregulated Immune Responses Can Result in Immunopathology

As discussed in the previous section, tissue damage and disease are common consequences of the immune response to viral infection, and these processes are often referred to as immune pathology. However, it is important to differentiate between damage that occurs during clearance or resolution of the viral infection vs instances where an overactive or dysregulated response results in tissue damage or disease that is disconnected from viral clearance or may even result in enhanced viral replication.[52,549] These processes can be driven by dysregulated innate immune or inflammatory responses,[267] as well as adaptive antibody- or T cell–dependent immune responses.

The adaptive immune system is frequently associated with virus-induced immunopathology, with B- and T-cell responses both contributing to disease. As discussed above, ADE of DENV by nonneutralizing heterologous antibody responses resulting in severe DENV-induced disease is an example of antibody-mediated immunopathology. Antibody immune complexes, which are generated during persistent infections with a number of viruses, including LCMV, HBV, and HCV, are another example of how antibody-mediated immunopathology can contribute to virus-induced disease. These complexes, which consist of virus-specific antibody bound to viral antigens, can be deposited in the kidney and induce complement activation and inflammation, leading to kidney damage.[118,198,356-358,383,499,500] T cells have also been demonstrated to play an important role in promoting immunopathologic tissue damage and disease. This is clearly illustrated by studies with LCMV. Although LCMV is noncytolytic for infected cells, intracranial infection of adult, immunocompetent mice induces fatal choriomeningitis (inflammation of the choroid plexus and meninges).[52,66,312] This disease process is mediated by virus-specific CD8 T cells killing virally infected cells in the CNS, and depletion of these cells protects against disease, demonstrating that the virus-specific T cells are driving virus-induced immunopathology.[52,66,278,335,550]

The host innate immune system can also contribute to immunopathology independently of adaptive immunity. Although the complement cascade plays a protective role against a wide variety of viruses, including WNV, VEEV, DENV, and Sindbis virus,[25,199,314] complement activation can also contribute to virus-induced immunopathology. RRV causes debilitating arthralgia and myalgia in humans, and the virus infects skeletal muscle and causes severe myositis in infected mice that is characterized by inflammatory cell infiltration and complement activation.[286,331,334] MBL binding to N-linked glycans on the RRV E2 glycoprotein expressed on the surface of virally infected muscle cells[185,186] activates complement and promotes cell killing by inflammatory macrophages leading to myositis and limb weakness.[333] Mice lacking key complement components, such as MBL or C3, are resistant to virus-induced disease, but viral replication and clearance are largely unaffected,[186,331,333] indicating that complement activation is driving nonprotective immunopathology in this model.

Other aspects of the innate immune system that normally play protective roles against viral infection can also contribute to virus-induced

immunopathology in certain situations, including the type I IFN response. As discussed above, type I IFN is normally essential for controlling many viral infections, however, in some situations type I IFNs can exacerbate disease and drive immune pathology. For example, mouse adapted strains of SARS-CoV-1 cause severe respiratory disease in BALB/c mice,[402] and disease pathogenesis is in part driven by type I IFN and inflammatory monocytes.[80] Type I IFN responses are delayed in SARS-CoV-1 infected mice and do not contribute to viral control,[147] possibly due to the wide variety of IFN evasion strategies employed by this virus.[145,146,260] Instead, type I IFN promotes the recruitment and activation of inflammatory monocytes into the SARS-CoV-1 infected lung, and loss of functional type I IFN receptor signaling or depletion of inflammatory monocytes results in decreased virus-induced disease and a reduction of virus-induced pathology within the lung.[80] Type I IFN signaling can also contribute to immune pathology during chronic viral infection. For example, type I IFN contributes to early control of LCMV infection, but during chronic infection type I IFN suppresses CD4 T-cell function through the upregulation of negative regulators, thus inhibiting T cell–mediated viral clearance to promote viral persistence.[476] Therefore, dysregulation of normally protective immune mediators can also promote immune pathology and/or interfere with viral clearance.

Virus-induced immunopathology is most often associated with direct interactions between the immune system and the virus (eg, ADE) or virally infected cells. However, there is also the possibility that viral infection will lead to autoimmunity where the host immune response targets self-antigens to induce diseases such as diabetes and neurologic disease. Although the immune system is tightly regulated to avoid the generation of autoreactive immune responses, viral infections might break tolerance to induce autoimmunity through several mechanisms, including molecular mimicry (cross-reactivity between viral antigens and host antigens), unmasking host antigens through the infection of immune privileged tissues or cells, or bystander reactivation of anergic autoreactive immune cells through the actions of virus-induced cytokines or inflammatory signals.[456] Experimental models provide support for molecular mimicry as a potential driver of autoimmunity. Studies of LCMV infection in transgenic mice expressing LCMV proteins in insulin-producing islet cells demonstrate that viral infection can trigger diabetes by triggering a T-cell response against the self-like antigens expressed in the transgenic pancreas.[354,359,360,513,514]

Importantly, while infection by a variety of viruses, including enteroviruses, herpesviruses, and influenza viruses has been associated with the onset of type I diabetes or other autoimmune diseases in humans,[456] a direct causal role for viruses initiating these disease processes through molecular mimicry or bystander activation has not yet been demonstrated. Although the role of viral infections in driving human autoimmunity is still largely undefined and requires additional investigation, there are clear examples of virus-induced autoimmunity, including Guillain-Barré syndrome (GBS), an autoimmune disease where the immune system targets the myelin sheath on peripheral nerves, that can result in limb weakness that can progress to breathing problems in severe cases. GBS onset is often associated with infection by a number of viruses, including CMV, VZV, EBV, HSV, SARS-CoV-2, WNV, ZIKV, and CHIKV.[255,405] While GBS induction by bacterial infections, such as *Campylobacter jejuni*, is associated with molecular mimicry where antibodies against bacterial lipo-oligosaccharides cross-react with gangliosides on peripheral motor neurons,[255,405] the mechanisms underlying virus-induced GBS are not currently understood. Therefore, further investigation into the pathogenesis of virus-induced GBS is required to understand how infection initiates autoimmune responses against peripheral nerves.

Viral Pathogenesis Research Going Forward

Over the past 40 years, the world has suffered three major pandemics (HIV, 2009 H1N1 Influenza A virus, and SARS-CoV-2), as well as multiple regional outbreaks and epidemics by emerging or reemerging viruses such as CHIKV, ZIKV, and Ebola virus. These viruses have caused significant human suffering, millions of deaths, trillions of dollars in economic loss, as well as significant social disruption and in some cases, the near collapse of public health systems. Importantly, these outbreaks appear to be occurring with increased frequency, and despite advances in vaccine and antiviral development, virus-induced disease represents an ongoing and growing threat to human health. Viral pathogenesis research to understand the mechanisms that drive virus-induced disease and the factors that determine an individual's susceptibility or resistance to a given viral infection represents a key part of the response to these viral threats. This information is essential for the development of safe and effective vaccines or therapies against existing virus-induced diseases, while also laying the groundwork to guide responses to newly emerging pathogens.

The viral pathogenesis field has benefited from advances in research areas such as molecular biology, synthetic biology, systems biology, functional genomics, and genome engineering to gain unprecedented insights into the biology and pathogenesis of a wide variety of viruses. The impact of these advances is illustrated by the rapid pace of research on SARS-CoV-2 pathogenesis, where many of the research areas and technologies discussed in this chapter, including epidemiology, clinical research, synthetic biology, systems biology, functional genomics technologies, and advanced animal models have informed the field's understanding of the pathogenesis of SARS-CoV-2-induced disease. In addition to providing important new insights into the pathogenesis of SARS-CoV-2-induced disease processes, this work, in combination with prior pathogenesis studies on the original SARS-CoV and MERS-CoV, has guided the rapid development and deployment of highly effective and safe SARS-CoV-2 vaccines and therapeutics. Given the threat posed by new emerging viruses, and the availability of increasingly powerful techniques for studying both the viral and the host side of the disease process, the viral pathogenesis field is likely to continue to thrive and provide new insights into virus-induced disease processes that inform public health responses to new and existing viral diseases.

REFERENCES

1. Abbas AK, Lichtman AH, Pillai S. Cellular and Molecular Immunology. 10th ed. Elsevier; 2021.

2. Ahmed R, Hahn CS, Somasundaram T, Villarete L, Matloubian M, Strauss JH. Molecular basis of organ-specific selection of viral variants during chronic infection. J Virol. 1991;65:4242-4247. doi:10.1128/JVI.65.8.4242-4247.1991

3. Ahmed R, Oldstone MB. Organ-specific selection of viral variants during chronic infection. J Exp Med. 1988;167:1719-1724. doi:10.1084/jem.167.5.1719

4. Aida T, Imahashi R, Tanaka K. Translating human genetics into mouse: the impact of ultra-rapid in vivo genome editing. Dev Growth Differ. 2014;56:34-45. doi:10.1111/dgd.12101

5. Akhrymuk I, Frolov I, Frolova EI. Sindbis virus infection causes cell death by nsP2-induced transcriptional shutoff or by nsP3-dependent translational shutoff. J Virol. 2018;92:e01388-18. doi:10.1128/JVI.01388-18

6. Akhrymuk I, Lukash T, Frolov I, Frolova EI. Novel mutations in nsP2 abolish Chikungunya virus-induced transcriptional shutoff and make the virus less cytopathic without affecting its replication rates. J Virol. 2019;93:e02062-18. doi:10.1128/JVI.02062-18

7. Akiyama BM, Laurence HM, Massey AR, et al. Zika virus produces noncoding RNAs using a multi-pseudoknot structure that confounds a cellular exonuclease. Science. 2016;354:1148-1152. doi:10.1126/science.aah3963

8. Alcami A, Smith GL. A soluble receptor for interleukin-1 beta encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. Cell. 1992;71:153-167. doi:10.1016/0092-8674(92)90274-g

9. Alcami A, Smith GL. A mechanism for the inhibition of fever by a virus. Proc Natl Acad Sci U S A. 1996;93:11029-11034. doi:10.1073/pnas.93.20.11029

10. Alghamdi IG, Hussain II, Almalki SS, et al. The pattern of Middle East respiratory syndrome coronavirus in Saudi Arabia: a descriptive epidemiological analysis of data from the Saudi Ministry of Health. Int J Gen Med. 2014;7:417-423. doi:10.2147/IJGM.S67061

11. Alkhatib G, Combadiere C, Broder CC, et al. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science. 1996;272:1955-1958. doi:10.1126/science.272.5270.1955

12. Allen TM, O'Connor DH, Jing P, et al. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature. 2000;407:386-390. doi:10.1038/35030124

13. Almanan M, Raynor J, Sholl A, et al. Tissue-specific control of latent CMV reactivation by regulatory T cells. PLoS Pathog. 2017;13:e1006507. doi:10.1371/journal.ppat.1006507

14. Almazan F, et al. Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. Proc Natl Acad Sci U S A. 2000;97:5516-5521. doi:10.1073/pnas.97.10.5516

15. Alzhanova D, Fruh K. Modulation of the host immune response by cowpox virus. Microbes Infect. 2010;12:900-909. doi:10.1016/j.micinf.2010.07.007

16. Ander SE, Diamond MS, Coyne CB. Immune responses at the maternal-fetal interface. Sci Immunol. 2019;4:eaat6114. doi:10.1126/sciimmunol.aat6114

17. Anderson EM, Goodwin EC, Verma A, et al. Seasonal human coronavirus antibodies are boosted upon SARS-CoV-2 infection but not associated with protection. Cell. 2021;184:1858-1864 e1810. doi:10.1016/j.cell.2021.02.010

18. Arjona A, Foellmer HG, Town T, et al. Abrogation of macrophage migration inhibitory factor decreases West Nile virus lethality by limiting viral neuroinvasion. J Clin Invest. 2007;117:3059-3066. doi:10.1172/JCI32218

19. Arnold CN, Barnes MJ, Berger M, et al. ENU-induced phenovariance in mice: inferences from 587 mutations. BMC Res Notes. 2012;5:577. doi:10.1186/1756-0500-5-577

20. Aronson JF, Grieder FB, Davis NL, et al. A single-site mutant and revertants arising in vivo define early steps in the pathogenesis of Venezuelan equine encephalitis virus. Virology. 2000;270:111-123. doi:10.1006/viro.2000.0241

21. Asano T, Boisson B, Onodi F, et al. X-linked recessive TLR7 deficiency in ~1% of men under 60 years old with life-threatening COVID-19. Sci Immunol. 2021;6:eabl4348. doi:10.1126/sciimmunol.abl4348

22. Ashbrook AW, Burrack KS, Silva LA, et al. Residue 82 of the Chikungunya virus E2 attachment protein modulates viral dissemination and arthritis in mice. J Virol. 2014;88:12180-12192. doi:10.1128/JVI.01672-14

23. Avadhanula V, Weasner BP, Hardy GG, Kumar JP, Hardy RW. A novel system for the launch of alphavirus RNA synthesis reveals a role for the Imd pathway in arthropod antiviral response. PLoS Pathog. 2009;5:e1000582. doi:10.1371/journal.ppat.1000582

24. Avila-Perez G, Nogales A, Park J-G, et al. A natural polymorphism in Zika virus NS2A protein responsible of virulence in mice. Sci Rep. 2019;9:19968. doi:10.1038/s41598-019-56291-4

25. Avirutnan P, Hauhart RE, Marovich MA, Garred P, Atkinson JP, Diamond MS. Complement-mediated neutralization of dengue virus requires mannose-binding lectin. mBio. 2011;2:e00276-11. doi:10.1128/mBio.00276-11

26. Baicus A. History of polio vaccination. World J Virol. 2012;1:108-114. doi:10.5501/wjv.v1.i4.108

27. Baiker A, Fabel K, Cozzio A, et al. Varicella-zoster virus infection of human neural cells in vivo. Proc Natl Acad Sci U S A. 2004;101:10792-10797. doi:10.1073/pnas.0404016101

28. Baldridge MT, Nice TJ, McCune BT, et al. Commensal microbes and interferon-lambda determine persistence of enteric murine norovirus infection. Science. 2015;347:266-269. doi:10.1126/science.1258025

29. Banach BS, Orenstein JM, Fox LM, Randell SH, Rowley AH, Baker SC. Human airway epithelial cell culture to identify new respiratory viruses: coronavirus NL63 as a model. J Virol Methods. 2009;156:19-26. doi:10.1016/j.jviromet.2008.10.022

30. Barouch DH, Kunstman J, Glowczwskie J, et al. Viral escape from dominant simian immunodeficiency virus epitope-specific cytotoxic T lymphocytes in DNA-vaccinated rhesus monkeys. J Virol. 2003;77:7367-7375. doi:10.1128/jvi.77.13.7367-7375.2003

31. Barouch DH, Kunstman J, Kuroda MJ, et al. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature. 2002;415:335-339. doi:10.1038/415335a

32. Barr KL, Vaidhyanathan V. Chikungunya in infants and children: is pathogenesis increasing? Viruses. 2019;11:294. doi:10.3390/v11030294

33. Barton E, Mandal P, Speck SH. Pathogenesis and host control of gammaherpesviruses: lessons from the mouse. Annu Rev Immunol. 2011;29:351-397. doi:10.1146/annurev-immunol-072710-081639

34. Bastard P, Gervais A, Le Voyer T, et al. Autoantibodies neutralizing type I IFNs are present in ~4% of uninfected individuals over 70 years old and account for ~20% of COVID-19 deaths. Sci Immunol. 2021;6:eabl4340. doi:10.1126/sciimmunol.abl4340

35. Bastard P, Rosen LB, Zhang Q, et al. Autoantibodies against type I IFNs in patients with life-threatening COVID-19. Science. 2020;370:eabd4585. doi:10.1126/science.abd4585

36. Beard CM, Benson RC Jr, Kelalis PP, Elveback LR, Kurland LT. The incidence and outcome of mumps orchitis in Rochester, Minnesota, 1935 to 1974. Mayo Clin Proc. 1977;52:3-7.

37. Becker Y. HSV-1 brain infection by the olfactory nerve route and virus latency and reactivation may cause learning and behavioral deficiencies and violence in children and adults: a point of view. Virus Genes. 1995;10:217-226. doi:10.1007/BF01701811

38. Bellido V, Perez A. COVID-19 and diabetes. J Clin Med. 2021;10. doi:10.3390/jcm10225341

39. Beral V, Peterman TA, Berkelman RL, Jaffe HW. Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection? Lancet. 1990;335:123-128. doi:10.1016/0140-6736(90)90001-l

40. Berger AK, Yi H, Kearns DB, Mainou BA. Bacteria and bacterial envelope components enhance mammalian reovirus thermostability. PLoS Pathog. 2017;13:e1006768. doi:10.1371/journal.ppat.1006768

41. Bernard E, Hamel R, Neyret A, et al. Human keratinocytes restrict chikungunya virus replication at a post-fusion step. Virology. 2015;476:1-10. doi:10.1016/j.virol.2014.11.013

42. Bernard KA, Klimstra WB, Johnston RE. Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. Virology. 2000;276:93-103. doi:10.1006/viro.2000.0546

43. Berns K, Wilson SJ, Panis M, et al. A large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature. 2004;428:431-437. doi:10.1038/nature02371

44. Bertolotti A, Thioune M, Abel S, et al. Prevalence of chronic chikungunya and associated risks factors in the French West Indies (La Martinique): a prospective cohort study. PLoS Negl Trop Dis. 2020;14:e0007327. doi:10.1371/journal.pntd.0007327

45. Billecocq A, Spiegel M, Vialat P, et al. NSs protein of Rift Valley fever virus blocks interferon production by inhibiting host gene transcription. J Virol. 2004;78:9798-9806. doi:10.1128/JVI.78.18.9798-9806.2004

46. Blondel B, Couderc T, Simonin Y, Gosselin AS, Guivel-Benhassine F. [Poliovirus and apoptosis]. Virologie (Montrouge). 2006;10:7-20. doi:10.1684/vir.2011.3461

47. Bloom JD. An experimentally determined evolutionary model dramatically improves phylogenetic fit. Mol Biol Evol. 2014;31:1956-1978. doi:10.1093/molbev/msu173

48. Blue CE, Spiller OB, Blackbourn DJ. The relevance of complement to virus biology. Virology. 2004;319:176-184. doi:10.1016/j.virol.2003.11.029

49. Blutt SE, Estes MK. Organoid models for infectious disease. Annu Rev Med. 2022;73:167-182. doi:10.1146/annurev-med-042320-023055

50. Bonavita CM, Cardin RD. Don't go breaking my heart: MCMV as a model for HCMV-associated cardiovascular diseases. Pathogens. 2021;10:619. doi:10.3390/pathogens10050619

51. Boon AC, deBeauchamp J, Hollmann A, et al. Host genetic variation affects resistance to infection with a highly pathogenic H5N1 influenza A virus in mice. J Virol. 2009;83:10417-10426. doi:10.1128/JVI.00514-09

52. Borrow P, Evans CF, Oldstone MB. Virus-induced immunosuppression: immune system-mediated destruction of virus-infected dendritic cells results in generalized immune suppression. J Virol. 1995;69:1059-1070. doi:10.1128/JVI.69.2.1059-1070.1995

53. Borrow P, Lewicki H, Wei X, et al. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat Med. 1997;3:205-211. doi:10.1038/nm0297-205

54. Bouloy M, Janzen C, Vialat P, et al. Genetic evidence for an interferon-antagonistic function of rift valley fever virus nonstructural protein NSs. J Virol. 2001;75:1371-1377. doi:10.1128/JVI.75.3.1371-1377.2001

55. Bournazos S, Vo HTM, Duong V. Antibody fucosylation predicts disease severity in secondary dengue infection. Science. 2021;372:1102-1105. doi:10.1126/science.abc730355a. Sagar M, Reifler K, Rossi M, et al. Recent endemic coronavirus infection is associated with less-severe COVID-19. J Clin Invest. 2021;131(1):e143380. doi: 10.1172/JCI143380

56. Bouvier NM, Lowen AC. Animal models for influenza virus pathogenesis and transmission. Viruses. 2010;2:1530-1563. doi:10.3390/v20801530

57. Bowden RJ, Simas JP, Davis AJ, Efstathiou S. Murine gammaherpesvirus 68 encodes tRNA-like sequences which are expressed during latency. J Gen Virol. 1997;78(Pt 7), 1675-1687. doi:10.1099/0022-1317-78-7-1675

58. Bradley KC, Finsterbusch K, Schnepf D, et al. Microbiota-driven tonic interferon signals in lung stromal cells protect from influenza virus infection. Cell Rep. 2019;28:245-256 e244. doi:10.1016/j.celrep.2019.05.105

59. Bradley MP, Nagamine CM. Animal models of zika virus. Comp Med. 2017;67:242-252.

60. Brasil P, Pereira Jr JP, Elisabeth Moreira M, et al. Zika virus infection in pregnant women in Rio de Janeiro. N Engl J Med. 2016;375:2321-2334. doi:10.1056/NEJMoa1602412

61. Brass AL, Dykxhoorn DM, Benita Y, et al. Identification of host proteins required for HIV infection through a functional genomic screen. Science. 2008;319:921-926. doi:10.1126/science.1152725

62. Bray M. The role of the Type I interferon response in the resistance of mice to filovirus infection. J Gen Virol. 2001;82:1365-1373. doi:10.1099/0022-1317-82-6-1365

63. Bray M, Davis K, Geisbert T, Schmaljohn C, Huggins J. A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. J Infect Dis. 1998;178:651-661. doi:10.1086/515386

64. Bridgen A, Elliott RM. Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs. Proc Natl Acad Sci U S A. 1996;93:15400-15404. doi:10.1073/pnas.93.26.15400

65. Bucardo F, Reyes Y, Morales M, et al. Association of genetic polymorphisms in DC-SIGN, toll-like receptor 3, and tumor necrosis factor alpha genes and the Lewis-negative phenotype with chikungunya infection and disease in Nicaragua. J Infect Dis. 2021;223:278-286. doi:10.1093/infdis/jiaa364

66. Buchmeier MJ, Welsh RM, Dutko FJ, Oldstone MB. The virology and immunobiology of lymphocytic choriomeningitis virus infection. Adv Immunol. 1980;30:275-331. doi:10.1016/s0065-2776(08)60197-2

67. Bulut O, Kilic G, Dominguez-Andres J. Immune memory in aging: a wide perspective covering microbiota, brain, metabolism, and epigenetics. Clin Rev Allergy Immunol. 2021. doi:10.1007/s12016-021-08905-x

68. Bumgarner R. Overview of DNA microarrays: types, applications, and their future. Curr Protoc Mol Biol. 2013;Chapter 22:Unit 22.1. doi:10.1002/0471142727.mb2201s101

69. Burge BW, Pfefferkorn ER. Conditional-lethal mutants of an animal virus: identification of two cistrons. Science. 1965;148:959-960. doi:10.1126/science.148.3672.959

70. Burke KP, Cox AL. Hepatitis C virus evasion of adaptive immune responses: a model for viral persistence. Immunol Res. 2010;47:216-227. doi:10.1007/s12026-009-8152-3

71. Burton TD, Eyre NS. Applications of deep mutational scanning in virology. Viruses. 2021;13:1020. doi:10.3390/v13061020

72. Cao-Lormeau VM, Blake A, Mons S, et al. Guillain-Barre syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. Lancet. 2016;387:1531-1539. doi:10.1016/S0140-6736(16)00562-6

73. Carbaugh DL, Zhou S, Sanders W, Moorman NJ, Swanstrom R, Lazear HM. Two genetic differences between closely related zika virus strains determine pathogenic outcome in mice. J Virol. 2020;94:e00618-20. doi:10.1128/JVI.00618-20

74. Carrington M, Martin MP. The impact of variation at the KIR gene cluster on human disease. Curr Top Microbiol Immunol. 2006;298:225-257. doi:10.1007/3-540- 27743-9_12

75. Casanova JL, Abel L. Human genetics of infectious diseases: a unified theory. EMBO J. 2007;26:915-922. doi:10.1038/sj.emboj.7601558

76. Casazza RL, Lazear HM, Miner JJ. Protective and pathogenic effects of interferon signaling during pregnancy. Viral Immunol. 2020;33:3-11. doi:10.1089/vim.2019.0076

77. Cello J, Paul AV, Wimmer E. Chemical synthesis of poliovirus cDNA: generation of infectious virus in the absence of natural template. Science. 2002;297:1016-1018. doi:10.1126/science.1072266

78. Centers for Disease Control. Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men—New York City and California. MMWR Morb Mortal Wkly Rep. 1981;30:305-308.

79. Chang Y, Cesarman E, Pessin MS, et al. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science. 1994;266:1865-1869. doi:10.1126/science.7997879

80. Channappanavar R, Fehr AR, Vijay R, et al. Dysregulated type I interferon and inflammatory monocyte-macrophage responses cause lethal pneumonia in SARS-CoV-infected mice. Cell Host Microbe. 2016;19:181-193. doi:10.1016/j.chom.2016.01.007

81. Channappanavar R, Fett C, Mack M, Ten Eyck PP, Meyerholz DK, Perlman S. Sex-based differences in susceptibility to severe acute respiratory syndrome coronavirus infection. J Immunol. 2017;198:4046-4053. doi:10.4049/jimmunol.1601896

82. Chapman EG, Costantino DA, Rabe JL, et al. The structural basis of pathogenic subgenomic flavivirus RNA (sfRNA) production. Science. 2014;344:307-310. doi:10.1126/science.1250897

83. Charles PC, Walters E, Margolis F, Johnston RE. Mechanism of neuroinvasion of Venezuelan equine encephalitis virus in the mouse. Virology. 1995;208:662-671. doi:10.1006/viro.1995.1197

84. Chau TN, Hieu NT, Anders KL, et al. Dengue virus infections and maternal antibody decay in a prospective birth cohort study of Vietnamese infants. J Infect Dis. 2009;200:1893-1900. doi:10.1086/648407

85. Chen N, Zhou M, Dong X, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020;395:507-513. doi:10.1016/S0140-6736(20)30211-7

86. Chetty A, Darby MG, Vornewald PM, et al. Il4ra-independent vaginal eosinophil accumulation following helminth infection exacerbates epithelial ulcerative pathology of HSV-2 infection. Cell Host Microbe. 2021;29:579-593 e575. doi:10.1016/j.chom.2021.02.004

87. Ch'ng TH, Enquist LW. Neuron-to-cell spread of pseudorabies virus in a compartmented neuronal culture system. J Virol.

2005;79:10875-10889. doi:10.1128/JVI.79.17.10875-10889.2005

88. Choi B, Choudhary MC, Regan J, et al. Persistence and evolution of SARS-CoV-2 in an immunocompromised host. N Engl J Med. 2020;383:2291-2293. doi:10.1056/NEJMc2031364

89. Chulanov V, Kostyusheva A, Brezgin S, et al. CRISPR screening: molecular tools for studying virus-host interactions. Viruses. 2021;13:2258. doi:10.3390/v13112258

90. Church DM, Goodstadt L, Hillier LW, et al. Lineage-specific biology revealed by a finished genome assembly of the mouse. PLoS Biol. 2009;7:e1000112. doi:10.1371/journal.pbio.1000112

91. Ciancanelli MJ, Abel L, Zhang SY, Casanova JL. Host genetics of severe influenza: from mouse Mx1 to human IRF7. Curr Opin Immunol. 2016;38:109-120. doi:10.1016/j.coi.2015.12.002

92. Ciancanelli MJ, Huang SXL, Luthra P, et al. Infectious disease. Life-threatening influenza and impaired interferon amplification in human IRF7 deficiency. Science. 2015;348:448-453. doi:10.1126/science.aaa1578

93. Clapham HE, Cummings DAT, Johansson MA. Immune status alters the probability of apparent illness due to dengue virus infection: evidence from a pooled analysis across multiple cohort and cluster studies. PLoS Negl Trop Dis. 2017;11:e0005926. doi:10.1371/journal.pntd.0005926

94. Clark LE, Clark SA, Lin CY, et al. VLDLR and ApoER2 are receptors for multiple alphaviruses. Nature. 2022;602:475-480. doi:10.1038/s41586-021-04326-0

95. Cockrell AS, Yount BL, Scobey T, et al. A mouse model for MERS coronavirus-induced acute respiratory distress syndrome. Nat Microbiol. 2016;2:16226. doi:10.1038/nmicrobiol.2016.226

96. Cohen SN, Chang AC, Boyer HW, Helling RB. Construction of biologically functional bacterial plasmids in vitro. Proc Natl Acad Sci U S A. 1973;70:3240-3244. doi:10.1073/pnas.70.11.3240

97. Collette NM, Lao VHI, Weilhammer DR, et al. Single amino acid mutations affect zika virus replication in vitro and virulence in vivo. Viruses. 2020;12:1295. doi:10.3390/v12111295

98. Connolly BM, Steele KE, Davis KJ, et al. Pathogenesis of experimental Ebola virus infection in guinea pigs. J Infect Dis. 1999;179(Suppl 1);S203-S217. doi:10.1086/514305

99. Cordeiro MT, Pena LJ, Brito CA, Gil LH, Marques ET. Positive IgM for Zika virus in the cerebrospinal fluid of 30 neonates with microcephaly in Brazil. Lancet. 2016;387:1811-1812. doi:10.1016/S0140-6736(16)30253-7

100. Crooke SN, Ovsyannikova IG, Poland GA, Kennedy RB. Immunosenescence and human vaccine immune responses. Immun Ageing. 2019;16:25. doi:10.1186/s12979-019-0164-9

101. Crozat K, Georgel P, Rutschmann S, et al. Analysis of the MCMV resistome by ENU mutagenesis. Mamm Genome. 2006;17:398-406. doi:10.1007/s00335-005-0164-2

102. Dag F, Dölken L, Holzki J, et al. Reversible silencing of cytomegalovirus genomes by type I interferon governs virus latency. PLoS Pathog. 2014;10:e1003962. doi:10.1371/journal.ppat.1003962

103. Davis NL, Fuller FJ, Dougherty WG, Olmsted RA, Johnston RE. A single nucleotide change in the E2 glycoprotein gene of Sindbis virus affects penetration rate in cell culture and virulence in neonatal mice. Proc Natl Acad Sci U S A. 1986;83:6771-6775. doi:10.1073/pnas.83.18.6771

104. Davis KL, Korom M, Morrison LA. Herpes simplex virus 2 ICP34.5 confers neurovirulence by regulating the type I interferon response. Virology. 2014;468-470:330-339. doi:10.1016/j.virol.2014.08.015

105. Dean M, Carrington M, Winkler C, et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. Science. 1996;273:1856-1862. doi:10.1126/science.273.5283.1856

106. de Castro-Jorge LA, de Carvalho RVH, Klein TM, et al. The NLRP3 inflammasome is involved with the pathogenesis of Mayaro virus. PLoS Pathog. 2019;15:e1007934. doi:10.1371/journal.ppat.1007934

107. de Lusignan S, Dorward J, Correa A, et al. Risk factors for SARS-CoV-2 among patients in the Oxford Royal College of General Practitioners Research and Surveillance Centre primary care network: a cross-sectional study. Lancet Infect Dis. 2020;20:1034-1042. doi:10.1016/S1473-3099(20)30371-6

108. de Wit E, Siegers JY, Cronin JM, et al. 1918 H1N1 influenza virus replicates and induces proinflammatory cytokine responses in extrarespiratory tissues of Ferrets. J Infect Dis. 2018;217:1237-1246. doi:10.1093/infdis/jiy003

109. Desai P, Diamond MS, Thackray LB. Helminth-virus interactions: determinants of coinfection outcomes. Gut Microbes. 2021;13:1961202. doi:10.1080/19490976.2021.1961202

110. Desai P, Janova H, White JP, et al. Enteric helminth coinfection enhances host susceptibility to neurotropic flaviviruses via a tuft cell-IL-4 receptor signaling axis. Cell. 2021;184:1214-1231 e1216. doi:10.1016/j.cell.2021.01.051

111. Desmyter J, Melnick JL, Rawls WE. Defectiveness of interferon production and of rubella virus interference in a line of African green monkey kidney cells (Vero). J Virol. 1968;2:955-961. doi:10.1128/JVI.2.10.955-961.1968

112. Dhakal S, Ruiz-Bedoya CA, Zhou R, et al. Sex differences in lung imaging and SARS-CoV-2 antibody responses in a COVID-19 Golden Syrian Hamster Model. *mBio*. 2021;12:e0097421. doi:10.1128/mBio.00974-21

113. Diaz MO, Ziemin S, Le Beau MM, et al. Homozygous deletion of the alpha- and beta 1-interferon genes in human leukemia and derived cell lines. Proc Natl Acad Sci U S A. 1988;85:5259-5263. doi:10.1073/pnas.85.14.5259

114. Dick GW, Kitchen SF, Haddow AJ. Zika virus. I. Isolations and serological specificity. Trans R Soc Trop Med Hyg. 1952;46:509-520. doi:10.1016/0035-9203(52)90042-4

115. Diebel KW, Claypool DJ, van Dyk LF. A conserved RNA polymerase III promoter required for gammaherpesvirus TMER transcription and microRNA processing. Gene. 2014;544:8-18. doi:10.1016/j.gene.2014.04.026

116. Diebel KW, Oko LM, Medina EM, et al. Gammaherpesvirus small noncoding RNAs are bifunctional elements that regulate infection and contribute to virulence in vivo. mBio. 2015;6:e01670-01614. doi:10.1128/mBio.01670-14

117. Dinnon KH III, Leist SR, Schäfer A, et al. A mouse-adapted model of SARS-CoV-2 to test COVID-19 countermeasures. Nature. 2020;586:560-566. doi:10.1038/s41586-020-2708-8

118. Dixon FJ, Oldstone MB, Tonietti G. Virus-induced immune-complex-type glomerulonephritis. Transplant Proc. 1969;1:945-948.

119. Donaldson EF, Lindesmith LC, Lobue AD, Baric RS. Viral shape-shifting: norovirus evasion of the human immune system. Nat Rev Microbiol. 2010;8:231-241. doi:10.1038/nrmicro2296

120. Donnelly CA, Ghani AC, Leung GM, et al. Epidemiological determinants of spread of causal agent of severe acute respiratory syndrome in Hong Kong. Lancet. 2003;361:1761-1766. doi:10.1016/S0140-6736(03)13410-1

121. Dubuisson J, Lustig S, Ruggli N, Akov Y, Rice CM. Genetic determinants of Sindbis virus neuroinvasiveness. J Virol. 1997;71:2636-2646. doi:10.1128/JVI.71.4.2636-2646.1997

122. Dudley JP, Lee NT. Disparities in age-specific morbidity and mortality from SARS-CoV-2 in China and the Republic of Korea. Clin Infect Dis. 2020;71:863-865. doi:10.1093/cid/ciaa354

123. Duffy MR, Chen T-H, Thane Hancock W, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med. 2009;360:2536-2543. doi:10.1056/NEJMoa0805715

124. Duggal NK, Emerman M. Evolutionary conflicts between viruses and restriction factors shape immunity. Nat Rev Immunol. 2012;12:687-695. doi:10.1038/nri3295

125. Dulbecco R. Production of plaques in monolayer tissue cultures by single particles of an animal virus. Proc Natl Acad Sci U S A. 1952;38:747-752. doi:10.1073/pnas.38.8.747

126. Dupuis S, Jouanguy E, Al-Hajjar S, et al. Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency. Nat Genet. 2003;33:388-391. doi:10.1038/ng1097

127. Ebihara H, Zivcec M, Gardner D, et al. A Syrian golden hamster model recapitulating ebola hemorrhagic fever. J Infect Dis. 2013;207:306-318. doi:10.1093/infdis/jis626

128. Enders JF, Weller TH, Robbins FC. Cultivation of the Lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. Science. 1949;109:85-87. doi:10.1126/science.109.2822.85

129. Endres MJ, Griot C, Gonzalez-Scarano F, Nathanson N. Neuroattenuation of an avirulent bunyavirus variant maps to the L RNA segment. J Virol. 1991;65:5465-5470. doi:10.1128/JVI.65.10.5465-5470.1991

130. Evans DT, O'Connor DH, Jing P, et al. Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef. Nat Med. 1999;5:1270-1276. doi:10.1038/15224

131. Farrag MA, Almajhdi FN. Human respiratory syncytial virus: role of innate immunity in clearance and disease progression. Viral Immunol. 2016;29:11-26. doi:10.1089/vim.2015.0098

132. Farrell HE, Vally H, Lynch DM, et al. Inhibition of natural killer cells by a cytomegalovirus MHC class I homologue in vivo. Nature. 1997;386:510-514. doi:10.1038/386510a0

133. Fei F, Smith JA, Cao L. Clinical laboratory characteristics in patients with suspected COVID-19: one single-institution experience. J Med Virol. 2021;93:1665-1671. doi:10.1002/jmv.26527

134. Feldman ER, Kara M, Oko LM, et al. A gammaherpesvirus noncoding RNA is essential for hematogenous dissemination and establishment of peripheral latency. mSphere. 2016;1:e00105-15. doi:10.1128/mSphere.00105-15

135. Feng E, Balint E, Poznanski SM, Ashkar AA, Loeb M. Aging and interferons: impacts on inflammation and viral disease outcomes. Cells. 2021;10:708. doi:10.3390/cells10030708

136. Fenner F. The Florey lecture, 1983. Biological control, as exemplified by smallpox eradication and myxomatosis. Proc R Soc Lond B Biol Sci. 1983;218:259-285. doi:10.1098/rspb.1983.0039

137. Fenner F, Marshall ID. A comparison of the virulence for European rabbits (*Oryctolagus cuniculus*) of strains of myxoma virus recovered in the field in Australia, Europe and America. J Hyg (Lond). 1957;55:149-191. doi:10.1017/s0022172400037098

138. Fenner F, Poole WE, Marshall ID, Dyce AL. Studies in the epidemiology of infectious myxomatosis of rabbits. VI. The experimental

introduction of the European strain of myxoma virus into Australian wild rabbit populations. J Hyg (Lond). 1957;55:192-206. doi:10.1017/s0022172400037104

139. Fenner F, Woodroofe GM. Changes in the virulence and antigenic structure of strains of myoma virus recovered from Australian wild rabbits between 1950 and 1964. Aust J Exp Biol Med Sci. 1965;43:359-370. doi:10.1038/icb.1965.69

140. Ferris MT, Aylor DL, Bottomly D, et al. Modeling host genetic regulation of influenza pathogenesis in the collaborative cross. PLoS Pathog. 2013;9:e1003196. doi:10.1371/journal.ppat.1003196

141. Fioravanti J, Di Lucia P, Magini D, et al. Effector CD8(+) T cell-derived interleukin-10 enhances acute liver immunopathology. J Hepatol. 2017;67:543-548. doi:10.1016/j.jhep.2017.04.020

142. Fisher MA, Lloyd ML. A review of murine cytomegalovirus as a model for human cytomegalovirus disease—do mice lie? Int J Mol Sci. 2020;22:214. doi:10.3390/ijms22010214

143. Foss S, Bottermann M, Jonsson A, Sandlie I, James LC, Andersen JT. TRIM21—from intracellular immunity to therapy. Front Immunol. 2019;10:2049. doi:10.3389/fimmu.2019.02049

144. Frenck R, Bernstein DI, Xia M, et al. Predicting susceptibility to norovirus GII.4 by use of a challenge model involving humans. J Infect Dis. 2012;206:1386-1393. doi:10.1093/infdis/jis514

145. Frieman M, Ratia K, Johnston RE, Mesecar AD, Baric RS. Severe acute respiratory syndrome coronavirus papain-like protease ubiquitin-like domain and catalytic domain regulate antagonism of IRF3 and NF-kappaB signaling. J Virol. 2009;83:6689-6705. doi:10.1128/JVI.02220-08

146. Frieman M, Yount B, Heise M, Kopecky-Bromberg SA, Palese P, Baric RS. Severe acute respiratory syndrome coronavirus ORF6 antagonizes STAT1 function by sequestering nuclear import factors on the rough endoplasmic reticulum/Golgi membrane. J Virol. 2007;81:9812-9824. doi:10.1128/JVI.01012-07

147. Frieman MB, Chen J, Morrison TE, et al. SARS-CoV pathogenesis is regulated by a STAT1 dependent but a type I, II and III interferon receptor independent mechanism. PLoS Pathog. 2010;6:e1000849. doi:10.1371/journal.ppat.1000849

148. Frolov I. Persistent infection and suppression of host response by alphaviruses. Arch Virol Suppl. 2004;(18):139-147. doi:10.1007/978-3-7091-0572-6_12

149. Frolova EI, et al. Roles of nonstructural protein nsP2 and Alpha/Beta interferons in determining the outcome of Sindbis virus infection. J Virol. 2002;76:11254-11264. doi:10.1128/jvi.76.22.11254-11264.2002

150. Fulcher ML, Gabriel S, Burns KA, Yankaskas JR, Randell SH. Well-differentiated human airway epithelial cell cultures. Methods Mol Med. 2005;107:183-206. doi:10.1385/1-59259-861-7:183

151. Gao SJ, Kingsley L, Li M, et al. KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma. Nat Med. 1996;2:925-928. doi:10.1038/nm0896-925

152. Garcia-Sastre A, Egorov A, Matassov D, et al. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology. 1998;252:324-330. doi:10.1006/viro.1998.9508

153. Gardner CL, Burke CW, Tesfay MZ, Glass PJ, Klimstra WB, Ryman KD. Eastern and Venezuelan equine encephalitis viruses differ in their ability to infect dendritic cells and macrophages: impact of altered cell tropism on pathogenesis. J Virol. 2008;82:10634-10646. doi:10.1128/JVI.01323-08

154. Gardner CL, Choi-Nurvitadhi J, Sun C, et al. Natural variation in the heparan sulfate binding domain of the eastern equine encephalitis virus E2 glycoprotein alters interactions with cell surfaces and virulence in mice. J Virol. 2013;87:8582-8590. doi:10.1128/JVI.00937-13

155. Gardner CL, Ebel GD, Ryman KD, Klimstra WB. Heparan sulfate binding by natural eastern equine encephalitis viruses promotes neurovirulence. Proc Natl Acad Sci U S A. 2011;108:16026-16031. doi:10.1073/pnas.1110617108

156. Gardner CL, Hritz J, Sun C, et al. Deliberate attenuation of chikungunya virus by adaptation to heparan sulfate-dependent infectivity: a model for rational arboviral vaccine design. PLoS Negl Trop Dis. 2014;8:e2719. doi:10.1371/journal.pntd.0002719

157. Gardner CL, Yin J, Burke CW, Klimstra WB, Ryman KD. Type I interferon induction is correlated with attenuation of a South American eastern equine encephalitis virus strain in mice. Virology. 2009;390:338-347. doi:10.1016/j.virol.2009.05.030

158. Gardner J, Anraku I, Le TT, et al. Chikungunya virus arthritis in adult wild-type mice. J Virol. 2010;84:8021-8032. doi:10.1128/JVI.02603-09

159. Garmashova N, Gorchakov R, Frolova E, Frolov I. Sindbis virus nonstructural proFatein nsP2 is cytotoxic and inhibits cellular transcription. J Virol. 2006;80:5686-5696. doi:10.1128/JVI.02739-05

160. Gaska JM, Ding Q, Ploss A. Mouse models for studying HCV vaccines and therapeutic antibodies. Methods Mol Biol. 2019;1911:481-503. doi:10.1007/978-1-4939-8976-8_33

161. Ge D, Fellay J, Thompson AJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009;461:399-401. doi:10.1038/nature 08309

162. Geisbert TW, Hensley LE, Larsen T, et al. Pathogenesis of Ebola hemorrhagic fever in cynomolgus macaques: evidence that dendritic cells are early and sustained targets of infection. Am J Pathol. 2003;163:2347-2370. doi:10.1016/S0002-9440(10)63591-2

163. Geisbert TW, Young HA, Jahrling PB, Davis KJ, Kagan E, Hensley LE. Mechanisms underlying coagulation abnormalities in ebola hemorrhagic fever: overexpression of tissue factor in primate monocytes/macrophages is a key event. J Infect Dis. 2003;188:1618-1629. doi:10.1086/379724

164. Geisbert TW, Young HA, Jahrling PB, et al. Pathogenesis of Ebola hemorrhagic fever in primate models: evidence that hemorrhage is not a direct effect of virus-induced cytolysis of endothelial cells. Am J Pathol. 2003;163:2371-2382. doi:10.1016/S0002-9440(10)63592-4

165. Giraldo D, Wilcox DR, Longnecker R. The type I interferon response and age-dependent susceptibility to herpes simplex virus infection. DNA Cell Biol. 2017;36:329-334. doi:10.1089/dna.2017.3668

166. Giurgea LT, Cervantes-Medina A, Walters K-A, et al. Sex differences in influenza: the challenge study experience. J Infect Dis. 2022;225(4):715-722. doi:10.1093/infdis/jiab422

167. Glass RI, Parashar UD, Estes MK. Norovirus gastroenteritis. N Engl J Med. 2009;361:1776-1785. doi:10.1056/NEJMra0804575

168. Gorman MJ, Caine EA, Zaitsev K, et al. An immunocompetent mouse model of Zika virus infection. Cell Host Microbe. 2018;23:672-685 e676. doi:10.1016/j.chom.2018.04.003

169. Gottwein E, Corcoran DL, Mukherjee N, et al. Viral microRNA targetome of KSHV-infected primary effusion lymphoma cell lines. Cell Host Microbe. 2011;10:515-526. doi:10.1016/j.chom.2011.09.012

170. Goulder PJ, Phillips RE, Colbert RA, et al. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat Med. 1997;3:212-217. doi:10.1038/nm0297-212

171. Graham JB, Swarts JL, Wilkins C, et al. A mouse model of chronic west nile virus disease. PLoS Pathog. 2016;12:e1005996. doi:10.1371/journal.ppat.1005996

172. Gralinski LE, Ferris MT, Aylor DL. Genome wide identification of SARS-CoV susceptibility loci using the collaborative cross. PLoS Genet. 2015;11:e1005504. doi:10.1371/journal.pgen.1005504

173. Grammatikos A, Donati M, Johnston SL, Gompels MM. Peripheral B cell deficiency and predisposition to viral infections: the paradigm of immune deficiencies. Front Immunol. 2021;12:731643. doi:10.3389/fimmu.2021.731643

174. Grau KR, Zhu S, Peterson ST, et al. The intestinal regionalization of acute norovirus infection is regulated by the microbiota via bile acid-mediated priming of type III interferon. Nat Microbiol. 2020;5:84-92. doi:10.1038/s41564-019-0602-7

175. Griffin DE. Immune responses during measles virus infection. Curr Top Microbiol Immunol. 1995;191:117-134. doi:10.1007/978-3-642-78621-1_8

176. Griffin DE. Virus-induced immune suppression. In: Nathanson N, et al., eds. Viral Pathogenesis. Lippincott-Raven; 1997:207-233.

177. Griffin DE. Recovery from viral encephalomyelitis: immune-mediated noncytolytic virus clearance from neurons. Immunol Res. 2010;47:123-133. doi:10.1007/s12026-009-8143-4

178. Griffin DE. Measles virus persistence and its consequences. Curr Opin Virol. 2020;41:46-51. doi:10.1016/j.coviro.2020.03.003

179. Griffiths P, Reeves M. Pathogenesis of human cytomegalovirus in the immunocompromised host. Nat Rev Microbiol. 2021;19:759-773. doi:10.1038/s41579-021-00582-z

180. Gromeier M, Wimmer E. Mechanism of injury-provoked poliomyelitis. J Virol. 1998;72:5056-5060. doi:10.1128/JVI.72.6.5056-5060.1998

181. Guan WJ, Ni Z-Y, Hu Y, et al. Clinical characteristics of coronavirus disease 2019 in China. N Engl J Med. 2020;382:1708-1720. doi:10.1056/NEJMoa2002032

182. Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R, Chisari FV. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity. 1996;4:25-36. doi:10.1016/s1074-7613(00)80295-2

183. Guidotti LG, Isogawa M, Chisari FV. Host-virus interactions in hepatitis B virus infection. Curr Opin Immunol. 2015;36:61-66. doi:10.1016/j.coi.2015.06.016

184. Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. Science. 1999;284:825-829. doi:10.1126/science.284.5415.825

185. Gunn BM, Jones JE, Shabman RS, et al. Ross River virus envelope glycans contribute to disease through activation of the host complement system. Virology. 2018;515:250-260. doi:10.1016/j.virol.2017.12.022

186. Gunn BM, Morrison TE, Whitmore AC, et al. Mannose binding lectin is required for alphavirus-induced arthritis/myositis. PLoS Pathog. 2012;8:e1002586. doi:10.1371/journal.ppat.1002586

187. Haase AT. Targeting early infection to prevent HIV-1 mucosal transmission. Nature. 2010;464:217-223. doi:10.1038/nature08757

188. Haller BL, Barkon ML, Li XY, et al. Brain- and intestine-specific variants of reovirus serotype 3 strain dearing are selected during chronic infection of severe combined immunodeficient mice. J Virol. 1995;69:3933-3937. doi:10.1128/JVI.69.6.3933-3937.1995

189. Haller BL, Barkon ML, Vogler GP, Virgin IV HW. Genetic mapping of reovirus virulence and organ tropism in severe combined immunodeficient mice: organ-specific virulence genes. J Virol. 1995;69:357-364. doi:10.1128/JVI.69.1.357-364.1995

190. Halstead SB, O'Rourke EJ. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. J Exp Med. 1977;146:201-217. doi:10.1084/jem.146.1.201

191. Hao L, Sakurai A, Watanabe T, et al. Drosophila RNAi screen identifies host genes important for influenza virus replication. Nature. 2008;454:890-893. doi:10.1038/nature07151

192. Hatta M, Gao P, Halfmann P, Kawaoka Y. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science. 2001;293:1840-1842. doi:10.1126/science.1062882

193. Hayes EB, Komar N, Nasci RS, et al. Epidemiology and transmission dynamics of West Nile virus disease. Emerg Infect Dis. 2005;11:1167-1173. doi:10.3201/eid1108. 050289a

194. Heise MT, Simpson DA, Johnston RE. A single amino acid change in nsP1 attenuates neurovirulence of the Sindbis-group alphavirus S.A.AR86. J Virol. 2000;74:4207-4213. doi:10.1128/jvi.74.9.4207-4213.2000

195. Hengel H, Lucin P, Jonjic S, Ruppert T, Koszinowski UH. Restoration of cytomegalovirus antigen presentation by gamma interferon combats viral escape. J Virol. 1994;68:289-297. doi:10.1128/JVI.68.1.289-297.1994

196. Hickman HD, Pierson TC. Zika in the brain: new models shed light on viral infection. Trends Mol Med. 2016;22:639-641. doi:10.1016/j.molmed.2016.06.004

197. Hirsch AJ. The use of RNAi-based screens to identify host proteins involved in viral replication. Future Microbiol. 2010;5:303-311. doi:10.2217/fmb.09.121

198. Hirsch MS, Allison AC, Harvey JJ. Immune complexes in mice infected neonatally with Moloney leukaemogenic and murine sarcoma viruses. Nature. 1969;223:739-740. doi:10.1038/223739a0

199. Hirsch RL, Griffin DE, Winkelstein JA. The role of complement in viral infections. II. the clearance of Sindbis virus from the bloodstream and central nervous system of mice depleted of complement. J Infect Dis. 1980;141:212-217. doi:10.1093/infdis/141.2.212

200. Hirsch VM, Olmsted RA, Murphey-Corb M, Purcell RH, Johnson PR. An African primate lentivirus (SIVsm) closely related to HIV-2. Nature. 1989;339:389-392. doi:10.1038/339389a0

201. Hohler T, Stradmann-Bellinghausen B, Starke R, et al. C4A deficiency and nonresponse to hepatitis B vaccination. J Hepatol. 2002;37:387-392. doi:10.1016/s0168-8278(02)00205-2

202. Honig A, Karussis D. Delayed-onset flaccid paralysis related to west Nile virus reactivation following treatment with rituximab: a case report. BMC Res Notes. 2014;7:852. doi:10.1186/1756-0500-7-852

203. Hou YJ, Okuda K, Edwards CE, et al. SARS-CoV-2 reverse genetics reveals a variable infection gradient in the respiratory tract. Cell. 2020;182:429-446 e414. doi:10.1016/j.cell.2020.05.042

204. Hrdy DB, Rubin DH, Fields BN. Molecular basis of reovirus neurovirulence: role of the M2 gene in avirulence. Proc Natl Acad Sci U S A. 1982;79:1298-1302. doi:10.1073/pnas.79.4.1298

205. Huang KY, Li CK-F, Clutterbuck E, et al. Virus-specific antibody secreting cell, memory B-cell, and sero-antibody responses in the human influenza challenge model. J Infect Dis. 2014;209:1354-1361. doi:10.1093/infdis/jit650

206. Huang W, Wang D, Yao YF. Understanding the pathogenesis of infectious diseases by single-cell RNA sequencing. Microb Cell. 2021;8:208-222. doi:10.15698/mic2021.09.759

207. Huet T, Cheynier R, Meyerhans A, Roelants G, Wain-Hobson S. Genetic organization of a chimpanzee lentivirus related to HIV-1. Nature. 1990;345:356-359. doi:10.1038/345356a0

208. Hutson AM, Atmar RL, Graham DY, Estes MK. Norwalk virus infection and disease is associated with ABO histo-blood group type. J Infect Dis. 2002;185:1335-1337. doi:10.1086/339883

209. Hyde JL, Chen R, Trobaugh DW, et al. The 5′ and 3′ ends of alphavirus RNAs—non-coding is not non-functional. Virus Res. 2015;206:99-107. doi:10.1016/j.virusres.2015.01.016

210. Hyde JL, Gardner CL, Kimura T, et al. A viral RNA structural element alters host recognition of nonself RNA. Science. 2014;343:783-787. doi:10.1126/science.1248465

211. Iannacone M, Guidotti LG. Immunobiology and pathogenesis of hepatitis B virus infection. Nat Rev Immunol. 2022;22:19-32. doi:10.1038/s41577-021-00549-4

212. Ichinohe T, Pang IK, Kumamoto Y, et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci U S A. 2011;108:5354-5359. doi:10.1073/pnas.1019378108

213. Ikegami T, Makino S. The pathogenesis of Rift Valley fever. Viruses. 2011;3:493-519. doi:10.3390/v3050493

International HIV Controllers Study; Pereyra F, Jia X, McLaren PJ, et al. The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. Science. 2010;330:1551-1557. doi:10.1126/science.1195271

215. International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome. Nature. 2004;431:931-945. doi:10.1038/nature03001

216. Ip WK, Chan KH, Law HKW, et al. Mannose-binding lectin in severe acute respiratory syndrome coronavirus infection. J Infect Dis.

2005;191:1697-1704. doi:10.1086/429631

217. Jackson CB, Farzan M, Chen B, Choe H. Mechanisms of SARS-CoV-2 entry into cells. Nat Rev Mol Cell Biol. 2022;23:3-20. doi:10.1038/s41580-021-00418-x

218. Jackson DA, Symons RH, Berg P. Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of *Escherichia coli*. Proc Natl Acad Sci U S A. 1972;69:2904-2909. doi:10.1073/pnas.69.10.2904

219. Jacob ST, Jacob ST, Crozier I, et al. Ebola virus disease. Nat Rev Dis Primers. 2020;6:13. doi:10.1038/s41572-020-0147-3

220. Jacob-Nascimento LC, Carvalho CX, Oliveira Silva MM, et al. Acute-phase levels of CXCL8 as risk factor for chronic arthralgia following chikungunya virus infection. Front Immunol. 2021;12:744183. doi:10.3389/fimmu.2021.744183

221. Jagger BW, Miner JJ, Cao B, et al. Gestational stage and IFN-lambda signaling regulate ZIKV infection in utero. Cell Host Microbe. 2017;22:366-376 e363. doi:10.1016/j.chom.2017.08.012

222. Jean CM, Honarmand S, Louie JK, Glaser CA. Risk factors for West Nile virus neuroinvasive disease, California, 2005. Emerg Infect Dis. 2007;13:1918-1920. doi:10.3201/eid1312.061265

223. Johnson WE, Desrosiers RC. Viral persistence: HIV's strategies of immune system evasion. Annu Rev Med. 2002;53:499-518. doi:10.1146/annurev.med.53.082901.104053

224. Jones MK, Watanabe M, Zhu S, et al. Enteric bacteria promote human and mouse norovirus infection of B cells. Science. 2014;346:755-759. doi:10.1126/science.1257147

225. Jones RA, Steckelberg A-L, Vicens Q, Szucs MJ, Akiyama BM, Kieft JS. Different tertiary interactions create the same important 3D features in a distinct flavivirus xrRNA. RNA. 2021;27:54-65. doi:10.1261/rna.077065.120

226. Jones SM, Feldmann H, Ströher U, et al. Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses. Nat Med. 2005;11:786-790. doi:10.1038/nm1258

227. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science. 2005;309:1577-1581. doi:10.1126/science.1113329

228. Jorgensen LVG. Zebrafish as a model for fish diseases in aquaculture. Pathogens. 2020;9:609. doi:10.3390/pathogens9080609

229. Kahan SM, Zajac AJ. Immune exhaustion: past lessons and new insights from lymphocytic choriomeningitis virus. Viruses. 2019;11:156. doi:10.3390/v11020156

230. Kamal RP, Katz JM, York IA. Molecular determinants of influenza virus pathogenesis in mice. Curr Top Microbiol Immunol. 2014;385:243-274. doi:10.1007/82_2014_388

231. Kamath RS, Fraser AG, Dong Y, et al. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature. 2003;421:231-237. doi:10.1038/nature01278

232. Kan B, Wang M, Jing H, et al. Molecular evolution analysis and geographic investigation of severe acute respiratory syndrome coronavirus-like virus in palm civets at an animal market and on farms. J Virol. 2005;79:11892-11900. doi:10.1128/JVI.79.18.11892-11900.2005

233. Kane M, Yadav SS, Bitzegeio J, et al. MX2 is an interferon-induced inhibitor of HIV-1 infection. Nature. 2013;502:563-566. doi:10.1038/nature12653

234. Kane M, Zang TM, Rihn SJ, et al. Identification of interferon-stimulated genes with antiretroviral activity. Cell Host Microbe. 2016;20:392-405. doi:10.1016/j.chom.2016.08.005

235. Kash JC, Tumpey TM, Proll SC, et al. Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. Nature. 2006;443:578-581. doi:10.1038/nature05181

236. Katano H, Ali MA, Patera AC, et al. Chronic active Epstein-Barr virus infection associated with mutations in perforin that impair its maturation. Blood. 2004;103:1244-1252. doi:10.1182/blood-2003-06-2171

237. Katze MG, Korth M, Law GL, Nathanson N. Viral Pathogenesis. 3rd ed. Academic Press; 2015.

238. Katzelnick LC, Gresh L, Elizabeth Halloran M, et al. Antibody-dependent enhancement of severe dengue disease in humans. Science. 2017;358:929-932. doi:10.1126/science.aan6836

239. Keane TM, Goodstadt L, Danecek P, et al. Mouse genomic variation and its effect on phenotypes and gene regulation. Nature. 2011;477:289-294. doi:10.1038/nature10413

240. Keroack M, Fields BN. Viral shedding and transmission between hosts determined by reovirus L2 gene. Science. 1986;232:1635-1638. doi:10.1126/science.3012780

241. Kerr PJ, Ghedin E, DePasse JV, et al. Evolutionary history and attenuation of myxoma virus on two continents. PLoS Pathog. 2012;8:e1002950. doi:10.1371/journal.ppat.1002950

242. Kestler H, Kodama T, Ringler D, et al. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science. 1990;248:1109-1112. doi:10.1126/science.2160735

243. Killingley B, Enstone JE, Greatorex J, et al. Use of a human influenza challenge model to assess person-to-person transmission: proof-of-concept study. J Infect Dis. 2012;205:35-43. doi:10.1093/infdis/jir701

244. Kim AS, Zimmerman O, Fox JM, et al. An evolutionary insertion in the Mxra8 receptor-binding site confers resistance to alphavirus infection and pathogenesis. Cell Host Microbe. 2020;27:428-440 e429. doi:10.1016/j.chom.2020.01.008

245. King CC, de Fries R, Kolhekar SR, Ahmed R. In vivo selection of lymphocyte-tropic and macrophage-tropic variants of lymphocytic choriomeningitis virus during persistent infection. J Virol. 1990;64:5611-5616. doi:10.1128/JVI.64.11.5611-5616.1990

246. Kliks SC, Nimmanitya S, Nisalak A, Burke DS. Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. Am J Trop Med Hyg. 1988;38:411-419. doi:10.4269/ajtmh.1988.38.411

247. Klimstra WB, Nangle EM, Smith MS, Yurochko AD, Ryman KD. DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. J Virol. 2003;77:12022-12032. doi:10.1128/jvi.77.22.12022-12032.2003

248. Klimstra WB, Ryman KD, Johnston RE. Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. J Virol. 1998;72:7357-7366. doi:10.1128/JVI.72.9.7357-7366.1998

249. Klingen Y, Conzelmann KK, Finke S. Double-labeled rabies virus: live tracking of enveloped virus transport. J Virol. 2008;82:237-245. doi:10.1128/JVI.01342-07

250. Knight AC, Montgomery SA, Fletcher CA, Baxter VK. Mouse models for the study of SARS-CoV-2 infection. Comp Med. 2021;71:383-397. doi:10.30802/AALAS-CM-21-000031

251. Knoll BM, Lindesmith LC, Yount BL, Baric RS, Marty FM. Resolution of diarrhea in an immunocompromised patient with chronic norovirus gastroenteritis correlates with constitution of specific antibody blockade titer. Infection. 2016;44:551-554. doi:10.1007/s15010-016-0875-1

252. Ko JY, Danielson ML, Town M, et al. Risk factors for coronavirus disease 2019 (COVID-19)-associated hospitalization: COVID-19-associated hospitalization surveillance network and behavioral risk factor surveillance system. Clin Infect Dis. 2021;72:e695-e703. doi:10.1093/cid/ciaa1419

253. Kobayashi T, Antar AAR, Boehme KW, et al. A plasmid-based reverse genetics system for animal double-stranded RNA viruses. Cell Host Microbe. 2007;1:147-157. doi:10.1016/j.chom.2007.03.003

254. Koenig S, Conley AJ, Brewah YA, et al. Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. Nat Med. 1995;1:330-336. doi:10.1038/nm0495-330

255. Koike H, Chiba A, Katsuno M. Emerging infection, vaccination, and Guillain-Barre syndrome: a review. Neurol Ther. 2021;10:523-537. doi:10.1007/s40120-021-00261-4

256. Koike S, Horie H, Sato Y, et al. Poliovirus-sensitive transgenic mice as a new animal model. Dev Biol Stand. 1993;78:101-107.

257. Koller BH, Smithies O. Altering genes in animals by gene targeting. Annu Rev Immunol. 1992;10:705-730. doi:10.1146/annurev.iy.10.040192.003421

258. Kollmus H, Pilzner C, Leist SR, et al. Of mice and men: the host response to influenza virus infection. Mamm Genome. 2018;29:446-470. doi:10.1007/s00335-018-9750-y

259. Konig R, Zhou Y, Elleder D, et al. Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. Cell. 2008;135:49-60. doi:10.1016/j.cell.2008. 07.032

260. Kopecky-Bromberg SA, Martinez-Sobrido L, Frieman M, Baric RA, Palese P. Severe acute respiratory syndrome coronavirus open reading frame (ORF) 3b, ORF 6, and nucleocapsid proteins function as interferon antagonists. J Virol. 2007;81:548-557. doi:10.1128/JVI.01782-06

261. Koyanagi N, Kawaguchi Y. Evasion of the cell-mediated immune response by alphaherpesviruses. Viruses. 2020;12:1354. doi:10.3390/v12121354

262. Krishnan MN, Ng A, Sukumaran B, et al. RNA interference screen for human genes associated with West Nile virus infection. Nature. 2008;455:242-245. doi:10.1038/nature07207

263. Kuhn JH, Li W, Choe H, Farzan M. Angiotensin-converting enzyme 2: a functional receptor for SARS coronavirus. Cell Mol Life Sci. 2004;61:2738-2743. doi:10.1007/s00018-004-4242-5

264. Kulcsar KA, Coleman CM, Beck SE, Frieman MB. Comorbid diabetes results in immune dysregulation and enhanced disease severity following MERS-CoV infection. JCI Insight. 2019;4. doi:10.1172/jci.insight.131774

265. Kumar N, Sharma S, Kumar R, et al. Host-directed antiviral therapy. Clin Microbiol Rev. 2020;33:e00168-19. doi:10.1128/CMR.00168-19

266. Kuroda M, Halfmann PJ, Hill-Batorski L, et al. Identification of interferon-stimulated genes that attenuate Ebola virus infection. Nat Commun. 2020;11:2953. doi:10.1038/s41467-020-16768-7

267. Kurt-Jones EA, Chan M, Zhou S, et al. Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. Proc Natl Acad Sci U S A. 2004;101:1315-1320. doi:10.1073/pnas.0308057100

268. Kuss SK, Best GT, Etheredge CA, et al. Intestinal microbiota promote enteric virus replication and systemic pathogenesis. Science. 2011;334:249-252. doi:10.1126/science.1211057

269. Kwan CK, Ernst JD. HIV and tuberculosis: a deadly human syndemic. Clin Microbiol Rev. 2011;24:351-376. doi:10.1128/CMR.00042-10

270. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. Nature. 2001;409:860-921. doi:10.1038/35057062

271. Lanford RE, Hildebrandt-Eriksen ES, Petri A, et al. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science. 2010;327:198-201. doi:10.1126/science.1178178

272. Lark T, Keck F, Narayanan A. Interactions of alphavirus nsP3 protein with host proteins. Front Microbiol. 2017;8:2652. doi:10.3389/fmicb.2017.02652

273. Lawrence T, Puel A, Reichenbach J, et al. Autosomal-dominant primary immunodeficiencies. Curr Opin Hematol. 2005;12:22-30. doi:10.1097/01.moh.0000149609.37309.0a

274. Lawson ND, Stillman EA, Whitt MA, Rose JK. Recombinant vesicular stomatitis viruses from DNA. Proc Natl Acad Sci U S A. 1995;92:4477-4481. doi:10.1073/pnas.92.10. 4477

275. Le May N, Dubaele S, De Santis LP, et al. TFIIH transcription factor, a target for the Rift Valley hemorrhagic fever virus. Cell. 2004;116:541-550. doi:10.1016/s0092-8674(04)00132-1

276. Legrand N, Ploss A, Balling R, et al. Humanized mice for modeling human infectious disease: challenges, progress, and outlook. Cell Host Microbe. 2009;6:5-9. doi:10.1016/j.chom.2009.06.006

277. Leist SR, Dinnon KH, Schäfer A, et al. A mouse-adapted SARS-CoV-2 induces acute lung injury and mortality in standard laboratory mice. Cell. 2020;183:1070-1085 e1012. doi:10.1016/j.cell.2020.09.050

278. Leist TP, Cobbold SP, Waldmann H, Aguet M, Zinkernagel RM. Functional analysis of T lymphocyte subsets in antiviral host defense. J Immunol. 1987;138:2278-2281.

279. Leong HN, Earnest A, Lim H-H, et al. SARS in Singapore—predictors of disease severity. Ann Acad Med Singap. 2006;35:326-331.

280. Levine B, Hardwick JM, Trapp BD, Crawford TO, Bollinger RC, Griffin DE. Antibody-mediated clearance of alphavirus infection from neurons. Science. 1991;254:856-860. doi:10.1126/science.1658936

281. Levine B, Huang Q, Isaacs JT, Reed JC, Griffin DE, Hardwick JM. Conversion of lytic to persistent alphavirus infection by the bcl-2 cellular oncogene. Nature. 1993;361:739-742. doi:10.1038/361739a0

282. Li K, Wohlford-Lenane CL, Channappanavar R, et al. Mouse-adapted MERS coronavirus causes lethal lung disease in human DPP4 knockin mice. Proc Natl Acad Sci U S A. 2017;114:E3119-E3128. doi:10.1073/pnas.1619109114

283. Li P, Wei Y, Mei M, et al. Integrative analysis of zika virus genome RNA structure reveals critical determinants of viral infectivity. Cell Host Microbe. 2018;24:875-886 e875. doi:10.1016/j.chom.2018.10.011

284. Li Z, Chen H, Jiao P, et al. Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. J Virol. 2005;79:12058-12064. doi:10.1128/JVI.79.18.12058-12064.2005

285. Lidbury BA, Rulli NE, Suhrbier A, et al. Macrophage-derived proinflammatory factors contribute to the development of arthritis and myositis after infection with an arthrogenic alphavirus. J Infect Dis. 2008;197:1585-1593. doi:10.1086/587841

286. Lidbury BA, Simeonovic C, Maxwell GE, Marshall ID, Hapel AJ. Macrophage-induced muscle pathology results in morbidity and mortality for Ross River virus-infected mice. J Infect Dis. 2000;181:27-34. doi:10.1086/315164

287. Liedtke W, Opalka B, Zimmermann CW, Lignitz E. Age distribution of latent herpes simplex virus 1 and varicella-zoster virus genome in human nervous tissue. J Neurol Sci. 1993;116:6-11. doi:10.1016/0022-510x(93)90082-a

288. Lim HK, Huang SXL, Chen J, et al. Severe influenza pneumonitis in children with inherited TLR3 deficiency. J Exp Med. 2019;216:2038-2056. doi:10.1084/jem.20181621

289. Lim PY, Behr MJ, Chadwick CM, Shi PY, Bernard KA. Keratinocytes are cell targets of West Nile virus in vivo. J Virol. 2011;85:5197-5201. doi:10.1128/JVI.02692-10

290. Lima GP, Rozenbaum D, Pimentel C, et al. Factors associated with the development of Congenital Zika Syndrome: a case-control study. BMC Infect Dis. 2019;19:277. doi:10.1186/s12879-019-3908-4

291. Lin S, Lin Y, Nery JR, et al. Comparison of the transcriptional landscapes between human and mouse tissues. Proc Natl Acad Sci U S A. 2014;111:17224-17229. doi:10.1073/pnas.1413624111

292. Lindesmith L, Moe C, Marionneau S, et al. Human susceptibility and resistance to Norwalk virus infection. Nat Med. 2003;9:548-553. doi:10.1038/nm860

293. Linnstaedt SD, Gottwein E, Skalsky RL, Luftig MA, Cullen BR. Virally induced cellular microRNA miR-155 plays a key role in B-cell immortalization by Epstein-Barr virus. J Virol. 2010;84:11670-11678. doi:10.1128/JVI.01248-10

294. Liu Y, Liu J, Plante KS, et al. The N501Y spike substitution enhances SARS-CoV-2 infection and transmission. Nature. 2022;602(7896):294-299. doi:10.1038/s41586-021-04245-0

295. Loh J, Chu DT, O'Guin AK, Yokoyama WM, Virgin HW IV. Natural killer cells utilize both perforin and gamma interferon to regulate murine cytomegalovirus infection in the spleen and liver. J Virol. 2005;79:661-667. doi:10.1128/JVI.79.1.661-667.2005

296. Loh J, Thomas DA, Revell PA, Ley TJ, Virgin HW IV. Granzymes and caspase 3 play important roles in control of gammaherpesvirus latency. J Virol. 2004;78:12519-12528. doi:10.1128/JVI.78.22.12519-12528.2004

297. Lopez J, Mommert M, Mouton W, et al. Early nasal type I IFN immunity against SARS-CoV-2 is compromised in patients with autoantibodies against type I IFNs. J Exp Med. 2021;218. doi:10.1084/jem.20211211

298. Louie JK, Acosta M, Winter K, et al. Factors associated with death or hospitalization due to pandemic 2009 influenza A(H1N1) infection in California. JAMA. 2009;302:1896-1902. doi:10.1001/jama.2009.1583

299. Lowy DR, Rands E, Chattopadhyay SK, Garon CF, Hager GL. Molecular cloning of infectious integrated murine leukemia virus DNA from infected mouse cells. Proc Natl Acad Sci U S A. 1980;77:614-618. doi:10.1073/pnas.77.1.614

300. Lucin P, Pavic I, Polic B, Jonjic S, Koszinowski UH. Gamma interferon-dependent clearance of cytomegalovirus infection in salivary glands. J Virol. 1992;66:1977-1984. doi:10.1128/JVI.66.4.1977-1984.1992

301. Luckow VA, Lee SC, Barry GF, Olins PO. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. J Virol. 1993;67:4566-4579. doi:10.1128/JVI.67.8.4566-4579.1993

302. Ma H, Kim AS, Kafai NM, et al. LDLRAD3 is a receptor for Venezuelan equine encephalitis virus. Nature. 2020;588:308-314. doi:10.1038/s41586-020-2915-3

303. Macadam AJ, et al. The 5′ noncoding region of the type 2 poliovirus vaccine strain contains determinants of attenuation and temperature sensitivity. Virology. 1991;181:451-458. doi:10.1016/0042-6822(91)90877-e

304. MacDonald GH, Johnston RE. Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis. J Virol. 2000;74:914-922. doi:10.1128/jvi.74.2.914-922.2000

305. Mallery DL, McEwan WA, Bidgood SR, Towers GJ, Johnson CM, James LC. Antibodies mediate intracellular immunity through tripartite motif-containing 21 (TRIM21). Proc Natl Acad Sci U S A. 2010;107:19985-19990. doi:10.1073/pnas.1014074107

306. Mangeat B, Turelli P, Caron G, Friedli M, Perrin L, Trono D. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. Nature. 2003;424:99-103. doi:10.1038/nature01709

307. Manivanh R, Mehrbach J, Knipe DM, Leib DA. Role of herpes simplex virus 1 gamma34.5 in the regulation of IRF3 signaling. J Virol. 2017;91:e01156-17. doi:10.1128/JVI. 01156-17

308. Martin EM. Studies on the RNA polymrase of some temperature-sensitive mutants of Semliki Forest virus. Virology. 1969;39:107-117. doi:10.1016/0042-6822(69)90352-3

309. Masopust D, Sivula CP, Jameson SC. Of mice, dirty mice, and men: using mice to understand human immunology. J Immunol. 2017;199:383-388. doi:10.4049/jimmunol.1700453

310. Matloubian M, Kolhekar SR, Somasundaram T, Ahmed R. Molecular determinants of macrophage tropism and viral persistence: importance of single amino acid changes in the polymerase and glycoprotein of lymphocytic choriomeningitis virus. J Virol. 1993;67:7340-7349. doi:10.1128/JVI.67.12.7340-7349.1993

311. Matloubian M, Somasundaram T, Kolhekar SR, Selvakumar R, Ahmed R. Genetic basis of viral persistence: single amino acid change in the viral glycoprotein affects ability of lymphocytic choriomeningitis virus to persist in adult mice. J Exp Med. 1990;172:1043-1048. doi:10.1084/jem.172.4.1043

312. McGavern DB, Homann D, Oldstone MB. T cells in the central nervous system: the delicate balance between viral clearance and disease. J Infect Dis. 2002;186(Suppl 2):S145-S151. doi:10.1086/344264

313. McLaughlin-Drubin ME, Meyers J, Munger K. Cancer associated human papillomaviruses. Curr Opin Virol. 2012;2:459-466. doi:10.1016/j.coviro.2012.05.004

314. Mehlhop E, Diamond MS. Protective immune responses against West Nile virus are primed by distinct complement activation pathways. J Exp Med. 2006;203:1371-1381. doi:10.1084/jem.20052388

315. Memoli MJ, Czajkowski L, Reed S, et al. Validation of the wild-type influenza A human challenge model H1N1pdMIST: an A(H1N1)pdm09 dose-finding investigational new drug study. Clin Infect Dis. 2015;60:693-702. doi:10.1093/cid/ciu924

316. Memoli MJ, Shaw PA, Han A, et al. Evaluation of antihemagglutinin and antineuraminidase antibodies as correlates of protection in an influenza A/H1N1 virus healthy human challenge model. mBio. 2016;7:e00417-16. doi:10.1128/mBio.00417-16

317. Menachery VD, Eisfeld AJ, Schäfer A, et al. MERS-CoV and H5N1 influenza virus antagonize antigen presentation by altering the epigenetic landscape. Proc Natl Acad Sci U S A. 2018;115:E1012-E1021. doi:10.1073/pnas.1706928115

318. Menachery VD, Eisfeld AJ, Schäfer A, et al. Pathogenic influenza viruses and coronaviruses utilize similar and contrasting approaches to control interferon-stimulated gene responses. mBio. 2014;5:e01174-14. doi:10.1128/mBio.01174-14

319. Menachery VD, Yount Jr BL, Debbink K, et al. A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence. Nat Med. 2015;21:1508-1513. doi:10.1038/nm.3985

320. Menachery VD, Yount Jr BL, Sims AC, et al. SARS-like WIV1-CoV poised for human emergence. Proc Natl Acad Sci U S A. 2016;113:3048-3053. doi:10.1073/pnas.1517719113

321. Meshram CD, Lukash T, Phillips AT, Akhrymuk I, Frolova EI, Frolov I. Lack of nsP2-specific nuclear functions attenuates chikungunya virus replication both in vitro and in vivo. Virology. 2019;534:14-24. doi:10.1016/j.virol.2019.05.016

322. Messerle M, Crnkovic I, Hammerschmidt W, Ziegler H, Koszinowski UH. Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. Proc Natl Acad Sci U S A. 1997;94:14759-14763. doi:10.1073/pnas.94.26.14759

323. Miller CJ, Marthas M. Simian immunodeficiency virus: animal models of disease. In: *Encyclopedia of Virology*. 3rd ed. Academic Press; 2008:594-603.

324. Miner JJ, Cook LE, Hong JP, et al. Therapy with CTLA4-Ig and an antiviral monoclonal antibody controls chikungunya virus arthritis. Sci Transl Med. 2017;9:eaah3438. doi:10.1126/scitranslmed.aah3438

325. Minor PD. Poliovirus. In: Nathanson N, et al., eds. Viral Pathogenesis. Lippincott-Raven; 1997:555-574.

326. Mitrovic M, Arapovic J, Traven L, Krmpotic A, Jonjic S. Innate immunity regulates adaptive immune response: lessons learned from studying the interplay between NK and CD8+ T cells during MCMV infection. Med Microbiol Immunol. 2012;201:487-495. doi:10.1007/s00430-012-0263-0

327. Mlcochova P, Kemp SA, Dhar MS, et al. SARS-CoV-2 B.1.617.2 Delta variant replication and immune evasion. Nature. 2021;599:114-119. doi:10.1038/s41586-021-03944-y

328. Monath TP. Treatment of yellow fever. Antiviral Res. 2008;78:116-124. doi:10.1016/j.antiviral.2007.10.009

329. Moore PS, Chang Y. Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and those without HIV infection. N Engl J Med. 1995;332:1181-1185. doi:10.1056/NEJM199505043321801

330. Mori I, Goshima F, Ito H, et al. The vomeronasal chemosensory system as a route of neuroinvasion by herpes simplex virus. Virology. 2005;334:51-58. doi:10.1016/j.virol.2005.01.023

331. Morrison TE, Fraser RJ, Smith PN, Mahalingam S, Heise MT. Complement contributes to inflammatory tissue destruction in a mouse model of Ross River virus-induced disease. J Virol. 2007;81:5132-5143. doi:10.1128/JVI.02799-06

332. Morrison TE, Oko L, Montgomery SA, et al. A mouse model of chikungunya virus-induced musculoskeletal inflammatory disease: evidence of arthritis, tenosynovitis, myositis, and persistence. Am J Pathol. 2011;178:32-40. doi:10.1016/j.ajpath.2010.11.018

333. Morrison TE, Simmons JD, Heise MT. Complement receptor 3 promotes severe ross river virus-induced disease. J Virol. 2008;82:11263-11272. doi:10.1128/JVI.01352-08

334. Morrison TE, Whitmore AC, Shabman RS, Lidbury BA, Mahalingam S, Heise MT. Characterization of Ross River virus tropism and virus-induced inflammation in a mouse model of viral arthritis and myositis. J Virol. 2006;80:737-749. doi:10.1128/JVI.80.2.737-749.2006

335. Moskophidis D, Cobbold SP, Waldmann H, Lehmann-Grube F. Mechanism of recovery from acute virus infection: treatment of lymphocytic choriomeningitis virus-infected mice with monoclonal antibodies reveals that Lyt-2+ T lymphocytes mediate clearance of virus and regulate the antiviral antibody response. J Virol. 1987;61:1867-1874. doi:10.1128/JVI.61.6.1867-1874.1987

336. Mouse Genome Sequencing Consortium. Waterston RH, Lindblad-Toh K, Birney E, et al. Initial sequencing and comparative analysis of the mouse genome. Nature. 2002;420:520-562. doi:10.1038/nature01262

337. Mullbacher A, Waring P, Tha Hla R, et al. Granzymes are the essential downstream effector molecules for the control of primary virus infections by cytolytic leukocytes. Proc Natl Acad Sci U S A. 1999;96:13950-13955. doi:10.1073/pnas.96.24.13950

338. Mullins JI, Hoover EA, Quackenbush SL, Donahue PR. Disease progression and viral genome variants in experimental feline leukemia virus-induced immunodeficiency syndrome. J Acquir Immune Defic Syndr (1988). 1991;4:547-557.

339. Murphy KM, Weaver C. Janeway's Immunobiology. 9th ed. W.W. Norton and Company; 2016.

340. Nagata N, Iwasaki T, Ami Y, et al. Comparison of neuropathogenicity of poliovirus type 3 in transgenic mice bearing the poliovirus receptor gene and cynomolgus monkeys. Vaccine. 2001;19:3201-3208. doi:10.1016/s0264-410x(01)00016-0

341. Nakayama E, Kato F, Tajima S, et al. Neuroinvasiveness of the MR766 strain of Zika virus in IFNAR−/− mice maps to prM residues conserved amongst African genotype viruses. PLoS Pathog. 2021;17:e1009788. doi:10.1371/journal.ppat.1009788

342. Nathanson N. Viral Pathogenesis and Immunity. Academic Press; 2007.

343. Nathanson N. Viral Pathogenesis. 1st ed. Raven; 1997.

344. Neumann G, Watanabe T, Ito H, et al. Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci U S A. 1999;96:9345-9350. doi:10.1073/pnas.96.16.9345

345. Nguyen-Van-Tam JS, Killingley B, Enstone J, et al. Minimal transmission in an influenza A (H3N2) human challenge-transmission model within a controlled exposure environment. PLoS Pathog. 2020;16:e1008704. doi:10.1371/journal.ppat.1008704

346. Nichols DB, De Martini W, Cottrell J. Poxviruses utilize multiple strategies to inhibit apoptosis. Viruses. 2017;9:215. doi:10.3390/v9080215

347. Nir Y, Beemer A, Goldwasser RA. West Nile virus infection in mice following exposure to a viral aerosol. Br J Exp Pathol. 1965;46:443-449.

348. Noll KE, Ferris MT, Heise MT. The Collaborative cross: a systems genetics resource for studying host-pathogen interactions. Cell Host

Microbe. 2019;25:484-498. doi:10.1016/j.chom.2019.03.009

349. Noll KE, Whitmore AC, West A, et al. Complex genetic architecture underlies regulation of influenza-A-virus-specific antibody responses in the collaborative cross. Cell Rep. 2020;31:107587. doi:10.1016/j.celrep.2020.107587

350. O'Brien KB, Vogel P, Duan S, et al. Impaired wound healing predisposes obese mice to severe influenza virus infection. J Infect Dis. 2012;205:252-261. doi:10.1093/infdis/jir729

351. O'Connor MA, Tisoncik-Go J, Lewis TB, et al. Early cellular innate immune responses drive Zika viral persistence and tissue tropism in pigtail macaques. Nat Commun. 2018;9:3371. doi:10.1038/s41467-018-05826-w

352. O'Donnell SM, Hansberger MW, Connolly JL, et al. Organ-specific roles for transcription factor NF-kappaB in reovirus-induced apoptosis and disease. J Clin Invest. 2005;115:2341-2350. doi:10.1172/JCI22428

353. Oh JE, Kim B-C, Chang D-H, et al. Dysbiosis-induced IL-33 contributes to impaired antiviral immunity in the genital mucosa. Proc Natl Acad Sci U S A. 2016;113:E762-E771. doi:10.1073/pnas.1518589113

354. Ohashi PS, Oehen S, Buerki K, et al. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. Cell. 1991;65:305-317. doi:10.1016/0092-8674(91)90164-t

355. Ohka S, Nomoto A. The molecular basis of poliovirus neurovirulence. Dev Biol (Basel). 2001;105:51-58.

356. Oldstone MB, Dixon FJ. Pathogenesis of chronic disease associated with persistent lymphocytic choriomeningitis viral infection. I. Relationship of antibody production to disease in neonatally infected mice. J Exp Med. 1969;129:483-505. doi:10.1084/jem.129. 3.483

357. Oldstone MB, Dixon FJ. Immune complex disease in chronic viral infections. J Exp Med. 1971;134:32-40.

358. Oldstone MB, Dixon FJ. Lactic dehydrogenase virus-induced immune complex type of glomerulonephritis. J Immunol. 1971;106:1260-1263.

359. Oldstone MB, Lewicki H, Borrow P, Hudrisier D, Gairin JE. Discriminated selection among viral peptides with the appropriate anchor residues: implications for the size of the cytotoxic T-lymphocyte repertoire and control of viral infection. J Virol. 1995;69;7423-7429. doi:10.1128/JVI.69.12.7423-7429.1995

360. Oldstone MB, Nerenberg M, Southern P, Price J, Lewicki H. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. Cell. 1991;65:319-331. doi:10.1016/0092-8674(91)90165-u

361. Orange JS. Natural killer cell deficiency. J Allergy Clin Immunol. 2013;132:515-525. doi:10.1016/j.jaci.2013.07.020

362. Orvedahl A, Alexander D, Tallóczy Z, et al. HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. Cell Host Microbe. 2007;1:23-35. doi:10.1016/j.chom.2006.12.001

363. Osada N, Kohara A, Yamaji T, et al. The genome landscape of the african green monkey kidney-derived vero cell line. DNA Res. 2014;21:673-683. doi:10.1093/dnares/dsu029

364. Osborne LC, Monticelli LA, Nice TJ, et al. Coinfection. Virus-helminth coinfection reveals a microbiota-independent mechanism of immunomodulation. Science. 2014;345:578-582. doi:10.1126/science.1256942

365. Owens M, Choe L, Rivera JE, Avila JD. West Nile virus neuroinvasive disease associated with rituximab therapy. J Neurovirol. 2020;26:611-614. doi:10.1007/s13365-020-00854-z

366. Oxford JS, Gill D. Unanswered questions about the 1918 influenza pandemic: origin, pathology, and the virus itself. Lancet Infect Dis. 2018;18:e348-e354. doi:10.1016/S1473-3099(18)30359-1

367. Paddison PJ, Silva JM, Conklin DS, et al. A resource for large-scale RNA-interference-based screens in mammals. Nature. 2004;428:427-431. doi:10.1038/nature02370

368. Pascal KE, Coleman CM, Mujica AO, et al. Pre- and postexposure efficacy of fully human antibodies against Spike protein in a novel humanized mouse model of MERS-CoV infection. Proc Natl Acad Sci U S A. 2015;112:8738-8743. doi:10.1073/pnas.1510830112

369. Patin NV, Peña-Gonzalez A, Hatt JK, Moe C, Kirby A, Konstantinidis KT. The role of the gut microbiome in resisting norovirus infection as revealed by a human challenge study mBio. 2020;11:e02634-20. doi:10.1128/mBio.02634-20

370. Peeters M, Honoré C, Huet T, et al. Isolation and partial characterization of an HIV-related virus occurring naturally in chimpanzees in Gabon. AIDS. 1989;3:625-630. doi:10.1097/00002030-198910000-00001

371. Peiris JS, Yuen KY, Osterhaus AD, Stohr K. The severe acute respiratory syndrome. N Engl J Med. 2003;349:2431-2441. doi:10.1056/NEJMra032498

372. Pereira RA, Simon MM, Simmons A. Granzyme A, a noncytolytic component of CD8(+) cell granules, restricts the spread of herpes simplex virus in the peripheral nervous systems of experimentally infected mice. J Virol. 2000;74:1029-1032. doi:10.1128/jvi.74.2.1029-1032.2000

373. Perez de Diego R, Sancho-Shimizu V, Lorenzo L, et al. Human TRAF3 adaptor molecule deficiency leads to impaired Toll-like receptor 3 response and susceptibility to herpes simplex encephalitis. Immunity. 2010;33:400-411. doi:10.1016/j.immuni.2010.08.014

374. Pesko K, Westbrook CJ, Mores CN, Lounibos LP, Reiskind MH. Effects of infectious virus dose and bloodmeal delivery method on susceptibility of *Aedes aegypti* and *Aedes albopictus* to chikungunya virus. J Med Entomol. 2009;46:395-399. doi:10.1603/033.046.0228

375. Petrilli CM, Jones SA, Yang J, et al. Factors associated with hospital admission and critical illness among 5279 people with coronavirus disease 2019 in New York City: prospective cohort study. BMJ. 2020;369:m1966. doi:10.1136/bmj.m1966

376. Picard C, Casanova JL. Novel primary immunodeficiencies. Adv Exp Med Biol. 2005;568:89-99. doi:10.1007/0-387-25342-4_6

377. Pijlman GP, Funk A, Kondratieva N, et al. A highly structured, nuclease-resistant, noncoding RNA produced by flaviviruses is required for pathogenicity. Cell Host Microbe. 2008;4:579-591. doi:10.1016/j.chom.2008.10.007

378. Pircher H, Moskophidis D, Rohrer U, Bürki K, Hengartner H, Zinkernagel RM. Viral escape by selection of cytotoxic T cell-resistant virus variants in vivo. Nature. 1990;346:629-633. doi:10.1038/346629a0

379. Piret J, Boivin G. Immunomodulatory strategies in herpes simplex virus encephalitis. Clin Microbiol Rev. 2020;33. doi:10.1128/CMR.00105-19

380. Plante JA, Liu Y, Liu J, et al. Spike mutation D614G alters SARS-CoV-2 fitness. Nature. 2021;592:116-121. doi:10.1038/s41586-020-2895-3

381. Pollara G, Kwan A, Newton PJ, et al. Dendritic cells in viral pathogenesis: protective or defective? Int J Exp Pathol. 2005;86:187-204. doi:10.1111/j.0959-9673.2005.00440.x

382. Polo JM, Davis NL, Rice CM, Huang HV, Johnston RE. Molecular analysis of Sindbis virus pathogenesis in neonatal mice by using virus recombinants constructed in vitro. J Virol. 1988;62:2124-2133. doi:10.1128/JVI.62.6.2124-2133.1988

383. Porter DD, Larsen AE, Porter HG. The pathogenesis of Aleutian disease of mink. I. In vivo viral replication and the host antibody response to viral antigen. J Exp Med. 1969;130:575-593. doi:10.1084/jem.130.3.575

384. Powers C, Fruh K. Rhesus CMV: an emerging animal model for human CMV. Med Microbiol Immunol. 2008;197:109-115. doi:10.1007/s00430-007-0073-y

385. Price-Haywood EG, Burton J, Fort D, Seoane L. Hospitalization and mortality among Black Patients and White Patients with Covid-19. N Engl J Med. 2020;382:2534-2543. doi:10.1056/NEJMsa2011686

386. Priess H, Eggers HJ. Synthesis and activity of RNA polymerase of a temperature sensitive poliovirus mutant at an elevated temperature. Nature. 1968;220:1047-1048. doi:10.1038/2201047a0

387. Puel A, Reichenbach J, Bustamante J, et al. The NEMO mutation creating the most-upstream premature stop codon is hypomorphic because of a reinitiation of translation. Am J Hum Genet. 2006;78:691-701. doi:10.1086/501532

388. Puerta-Guardo H, Glasner DR, Espinosa DA, et al. Flavivirus NS1 triggers tissue-specific vascular endothelial dysfunction reflecting disease tropism. Cell Rep. 2019;26:1598-1613 e1598. doi:10.1016/j.celrep.2019.01.036

389. Quaresma JA, Barros VLRS, Pagliari C, et al. Revisiting the liver in human yellow fever: virus-induced apoptosis in hepatocytes associated with TGF-beta, TNF-alpha and NK cells activity. Virology. 2006;345:22-30. doi:10.1016/j.virol.2005.09.058

390. Quaresma JA, Barros VLRS, Pagliari C, et al. Hepatocyte lesions and cellular immune response in yellow fever infection. Trans R Soc Trop Med Hyg. 2007;101:161-168. doi:10.1016/j.trstmh.2006.02.019

391. Racaniello VR. One hundred years of poliovirus pathogenesis. Virology. 2006;344:9-16. doi:10.1016/j.virol.2005.09.015

392. Racaniello VR, Baltimore D. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science. 1981;214:916-919. doi:10.1126/science.6272391

393. Racaniello VR, Ren R. Poliovirus biology and pathogenesis. Curr Top Microbiol Immunol. 1996;206:305-325. doi:10.1007/978-3-642-85208-4_15

394. Ramoz N, Rueda L-A, Bouadjar B, Montoya L-S, Orth G, Favre M. Mutations in two adjacent novel genes are associated with epidermodysplasia verruciformis. Nat Genet. 2002;32:579-581. doi:10.1038/ng1044

395. Rasmussen AL, Okumura A, Ferris MT, et al. Host genetic diversity enables Ebola hemorrhagic fever pathogenesis and resistance. Science. 2014;346:987-991. doi:10.1126/science.1259595

396. RECOVERY Collaborative Group; Horby P, Lim WS, Emberson JR, et al. Dexamethasone in hospitalized patients with Covid-19. N Engl J Med. 2021;384:693-704. doi:10.1056/NEJMoa2021436

397. Reddehase MJ, Mutter W, Munch K, Buhring HJ, Koszinowski UH. CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. J Virol. 1987;61:3102-3108. doi:10.1128/JVI.61.10.3102-3108.1987

398. Reiskind MH, Pesko K, Westbrook CJ, Mores CN. Susceptibility of Florida mosquitoes to infection with chikungunya virus. Am J Trop Med Hyg. 2008;78:422-425.

399. Ren R, Racaniello VR. Poliovirus spreads from muscle to the central nervous system by neural pathways. J Infect Dis. 1992;166:747-752. doi:10.1093/infdis/166.4.747

400. Rice CM, Levis R, Strauss JH, Huang HV. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. J Virol. 1987;61:3809-3819. doi:10.1128/JVI.61.12.3809-3819.1987

401. Roark HK, Jenks JA, Permar SR, Schleiss MR. Animal models of congenital cytomegalovirus transmission: implications for vaccine

development. J Infect Dis. 2020;221:S60-S73. doi:10.1093/infdis/jiz484

402. Roberts A, Deming D, Paddock CD, et al. A mouse-adapted SARS-coronavirus causes disease and mortality in BALB/c mice. PLoS Pathog. 2007;3:e5. doi:10.1371/journal.ppat.0030005

403. Roberts A, Vogel L, Guarner J, et al. Severe acute respiratory syndrome coronavirus infection of golden Syrian hamsters. J Virol. 2005;79:503-511. doi:10.1128/JVI.79.1.503-511.2005

404. Rockx B, Sheahan T, Donaldson E, et al. Synthetic reconstruction of zoonotic and early human severe acute respiratory syndrome coronavirus isolates that produce fatal disease in aged mice. J Virol. 2007;81:7410-7423. doi:10.1128/JVI.00505-07

405. Rodriguez Y, Rojas M, Pacheco Y, et al. Guillain-Barre syndrome, transverse myelitis and infectious diseases. Cell Mol Immunol. 2018;15:547-562. doi:10.1038/cmi.2017.142

406. Rogers KJ, Shtanko O, Vijay R, et al. Acute plasmodium infection promotes interferon-gamma-dependent resistance to Ebola virus infection. Cell Rep. 2020;30:4041-4051 e4044. doi:10.1016/j.celrep.2020.02.104

407. Rolot M, Dougall AM, Chetty A, et al. Helminth-induced IL-4 expands bystander memory CD8(+) T cells for early control of viral infection. Nat Commun. 2018;9:4516. doi:10.1038/s41467-018-06978-5

408. Rose PP, Hanna SL, Spiridigliozzi A, et al. Natural resistance-associated macrophage protein is a cellular receptor for sindbis virus in both insect and mammalian hosts. Cell Host Microbe. 2011;10:97-104. doi:10.1016/j.chom.2011.06.009

409. Rosenberg BR, Freije CA, Imanaka N, et al. Genetic variation at IFNL4 influences extrahepatic interferon-stimulated gene expression in chronic HCV patients. J Infect Dis. 2018;217:650-655. doi:10.1093/infdis/jix593

410. Rosendo Machado S, van der Most T, Miesen P. Genetic determinants of antiviral immunity in dipteran insects—compiling the experimental evidence. Dev Comp Immunol. 2021;119:104010. doi:10.1016/j.dci.2021.104010

411. Rosenke K, Adjemian J, Munster VJ, et al. Plasmodium parasitemia associated with increased survival in Ebola virus-infected patients. Clin Infect Dis. 2016;63:1026-1033. doi:10.1093/cid/ciw452

412. Ruffner MA, Sullivan KE, Henrickson SE. Recurrent and sustained viral infections in primary immunodeficiencies. Front Immunol. 2017;8:665. doi:10.3389/fimmu.2017.00665

413. Rulli NE, Guglielmotti A, Mangano G, et al. Amelioration of alphavirus-induced arthritis and myositis in a mouse model by treatment with bindarit, an inhibitor of monocyte chemotactic proteins. Arthritis Rheum. 2009;60:2513-2523. doi:10.1002/art.24682

414. Ryman KD, Gardner CL, Burke CW, Meier KC, Thompson JM, Klimstra WB. Heparan sulfate binding can contribute to the neurovirulence of neuroadapted and nonneuroadapted Sindbis viruses. J Virol. 2007;81:3563-3573. doi:10.1128/JVI.02494-06

415. Ryman KD, Klimstra WB, Nguyen KB, Biron CA, Johnston RE. Alpha/beta interferon protects adult mice from fatal Sindbis virus infection and is an important determinant of cell and tissue tropism. J Virol. 2000;74:3366-3378. doi:10.1128/jvi.74.7.3366-3378.2000

416. Sabin AB, Ramos-Alvarez M, Alvarez-Amezquita J, et al. Landmark article Aug 6, 1960: Live, orally given poliovirus vaccine. Effects of rapid mass immunization on population under conditions of massive enteric infection with other viruses. By Albert B. Sabin, Manuel Ramos-Alvarez, Jose Alvarez-Amezquita, William Pelon, Richard H. Michaels, Ilya Spigland, Meinrad A. Koch, Joan M. Barnes, and Johng S. Rhim. JAMA. 1984;251:2988-2993. doi:10.1001/jama.251.22.2988

417. Salleh MZ, Derrick JP, Deris ZZ. Structural evaluation of the spike glycoprotein variants on SARS-CoV-2 transmission and immune evasion. Int J Mol Sci. 2021;22:7425. doi:10.3390/ijms22147425

418. Samson M, Libert F, Doranz BJ, et al. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature. 1996;382:722-725. doi:10.1038/382722a0

419. Sancho-Shimizu V, de Diego RP, Lorenzo L, et al. Herpes simplex encephalitis in children with autosomal recessive and dominant TRIF deficiency. J Clin Invest. 2011;121:4889-4902. doi:10.1172/JCI59259

420. Sangkawibha N, Rojanasuphot S, Ahandrik S, et al. Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. Am J Epidemiol. 1984;120:653-669. doi:10.1093/oxfordjournals.aje.a113932

421. Santoriello C, Zon LI. Hooked! Modeling human disease in zebrafish. J Clin Invest. 2012;122:2337-2343. doi:10.1172/JCI60434

422. Sarkar S, Heise MT. Mouse models as resources for studying infectious diseases. Clin Ther. 2019;41:1912-1922. doi:10.1016/j.clinthera.2019.08.010

423. Sausen DG, Reed KM, Bhutta MS, Gallo ES, Borenstein R. Evasion of the host immune response by betaherpesviruses. Int J Mol Sci. 2021;22:7503. doi:10.3390/ijms22147503

424. Schaap A, Fortin J-F, Sommer M, et al. T-cell tropism and the role of ORF66 protein in pathogenesis of varicella-zoster virus infection. J Virol. 2005;79:12921-12933. doi:10.1128/JVI.79.20.12921-12933.2005

425. Schafer A, Brooke CB, Whitmore AC, Johnston RE. The role of the blood-brain barrier during Venezuelan equine encephalitis virus infection. J Virol. 2011;85:10682-10690. doi:10.1128/JVI.05032-11

426. Schaffer P, Vonka V, Lewis R, Benyesh-Melnick M. Temperature-sensitive mutants of herpes simplex virus. Virology. 1970;42:1144-1146. doi:10.1016/0042-6822(70)90364-8

427. Schaffer PA, Aron GM, Biswal N, Benyesh-Melnick M. Temperature-sensitive mutants of herpes simplex virus type 1: isolation, complementation and partial characterization. Virology. 1973;52:57-71. doi:10.1016/0042-6822(73)90398-x

428. Scheer S, Krempl C, Kallfass C, et al. S mansoni bolsters anti-viral immunity in the murine respiratory tract. PLoS One. 2014;9:e112469. doi:10.1371/journal.pone.0112469

429. Schlesinger MJ, Schlesinger S. Large-molecular-weight precursors of sindbis virus proteins. J Virol. 1973;11:1013-1016. doi:10.1128/JVI.11.6.1013-1016.1973

430. Schneider CA, Calvo E, Peterson KE. Arboviruses: how saliva impacts the journey from vector to host. Int J Mol Sci. 2021;22:9173. doi:10.3390/ijms22179173

431. Schnell MJ, Mebatsion T, Conzelmann KK. Infectious rabies viruses from cloned cDNA. EMBO J. 1994;13:4195-4203.

432. Schoggins JW, MacDuff DA, Imanaka N, et al. Pan-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity. Nature. 2014;505:691-695. doi:10.1038/nature12862

433. Schoggins JW, Rice CM. Interferon-stimulated genes and their antiviral effector functions. Curr Opin Virol. 2011;1:519-525. doi:10.1016/j.coviro.2011.10.008

434. Schoggins JW, Wilson SJ, Panis M, et al. A diverse range of gene products are effectors of the type I interferon antiviral response. Nature. 2011;472:481-485. doi:10.1038/nature09907

435. Schrofelbauer B, Yu Q, Landau NR. New insights into the role of Vif in HIV-1 replication. AIDS Rev. 2004;6:34-39.

436. Scobey T, Yount BL, Sims AC, et al. Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus. Proc Natl Acad Sci U S A. 2013;110:16157-16162. doi:10.1073/pnas.1311542110

437. Scully EP, Haverfield J, Ursin RL, Tannenbaum C, Klein SL. Considering how biological sex impacts immune responses and COVID-19 outcomes. Nat Rev Immunol. 2020;20:442-447. doi:10.1038/s41577-020-0348-8

438. Sellin CI, Horvat B. Current animal models: transgenic animal models for the study of measles pathogenesis. Curr Top Microbiol Immunol. 2009;330:111-127. doi:10.1007/978-3-540-70617-5_6

439. Shah PS, Link N, Jang GM, et al. Comparative flavivirus-host protein interaction mapping reveals mechanisms of dengue and zika virus pathogenesis. Cell. 2018;175:1931-1945 e1918. doi:10.1016/j.cell.2018.11.028

440. Shan C, Xia H, Haller SL, et al. A Zika virus envelope mutation preceding the 2015 epidemic enhances virulence and fitness for transmission. Proc Natl Acad Sci U S A. 2020;117:20190-20197. doi:10.1073/pnas.2005722117

441. Sharma S, Thomas PG. The two faces of heterologous immunity: protection or immunopathology. J Leukoc Biol. 2014;95:405-416. doi:10.1189/jlb.0713386

442. Sharp PM, Hahn BH. The evolution of HIV-1 and the origin of AIDS. Philos Trans R Soc Lond B Biol Sci. 2010;365:2487-2494. doi:10.1098/rstb.2010.0031

443. Sheahan T, Rockx B, Donaldson E, et al. Mechanisms of zoonotic severe acute respiratory syndrome coronavirus host range expansion in human airway epithelium. J Virol. 2008;82:2274-2285. doi:10.1128/JVI.02041-07

444. Shinya K, Hamm S, Hatta M, Ito H, Ito T, Kawaoka Y. PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology. 2004;320:258-266. doi:10.1016/j.virol.2003.11.030

445. Shou S, Liu M, Yang Y, et al. Animal models for COVID-19: hamsters, mouse, ferret, mink, tree shrew, and non-human primates. Front Microbiol. 2021;12:626553. doi:10.3389/fmicb.2021.626553

446. Shrestha B, Samuel MA, Diamond MS. CD8+ T cells require perforin to clear West Nile virus from infected neurons. J Virol. 2006;80:119-129. doi:10.1128/JVI.80.1.119-129.2006

447. Sia SF, Yan L-M, Chin AWH, et al. Pathogenesis and transmission of SARS-CoV-2 in golden hamsters. Nature. 2020;583:834-838. doi:10.1038/s41586-020-2342-5

448. Sigel MM. Influence of age on susceptibility to virus infections with particular reference to laboratory animals. Annu Rev Microbiol. 1952;6:247-280. doi:10.1146/annurev.mi.06.100152.001335

449. Simmons A, Aluvihare V, McMichael A. Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators. Immunity. 2001;14:763-777. doi:10.1016/s1074-7613(01)00158-3

450. Simmons CP, Chau TNB, Thuy TT, et al. Maternal antibody and viral factors in the pathogenesis of dengue virus in infants. J Infect Dis. 2007;196:416-424. doi:10.1086/519170

451. Simonnet A, Chetboun M, Poissy J, et al. High prevalence of obesity in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) requiring invasive mechanical ventilation. Obesity (Silver Spring). 2020;28:1195-1199. doi:10.1002/oby.22831

452. Siragam V, Wong G, Qiu XG. Animal models for filovirus infections. Zool Res. 2018;39:15-24. doi:10.24272/j.issn.2095-8137.2017.053

453. Skalsky RL, Cullen BR. Viruses, microRNAs, and host interactions. Annu Rev Microbiol. 2010;64:123-141. doi:10.1146/annurev.micro.112408.134243

454. Skarnes WC, Rosen B, West AP, et al. A conditional knockout resource for the genome-wide study of mouse gene function. Nature. 2011;474:337-342. doi:10.1038/nature10163

455. Slonchak, A, Khromykh AA. Subgenomic flaviviral RNAs: what do we know after the first decade of research. Antiviral Res. 2018;159:13-25. doi:10.1016/j.antiviral.2018.09.006

456. Smatti MK, Cyprian FS, Nasrallah GK, Al Thani AA, Almishal RO, Yassine HM. Viruses and autoimmunity: a review on the potential interaction and molecular mechanisms. Viruses. 2019;11:762. doi:10.3390/v11080762

457. Smiley JR. Construction in vitro and rescue of a thymidine kinase-deficient deletion mutation of herpes simplex virus. Nature. 1980;285:333-335. doi:10.1038/285333a0

458. Smith AG, Sheridan PA, Harp JB, Beck MA. Diet-induced obese mice have increased mortality and altered immune responses when infected with influenza virus. J Nutr. 2007;137:1236-1243. doi:10.1093/jn/137.5.1236

459. Smith GL, Benfield CTO, de Motes CM, et al. Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity. J Gen Virol. 2013;94:2367-2392. doi:10.1099/vir.0.055921-0

460. Sparks H, Monogue B, Akiyama B, Kieft J, Beckham JD. Disruption of Zika virus xrRNA1-dependent sfRNA1 production results in tissue-specific attenuated viral replication. Viruses. 2020;12:1177. doi:10.3390/v12101177

461. Speck SH. Regulation of EBV latency-associated gene expression. In: Robertson ES, ed. Epstein-Barr Virus. The Cromwell Press; 2005.

462. Spriggs DR, Bronson RT, Fields BN. Hemagglutinin variants of reovirus type 3 have altered central nervous system tropism. Science. 1983;220:505-507. doi:10.1126/science.6301010

463. Srivastava A, Morgan AP, Najarian ML, et al. Genomes of the mouse collaborative cross. Genetics. 2017;206:537-556. doi:10.1534/genetics.116.198838

464. Starr TN, Greaney AJ, Addetia A, et al. Prospective mapping of viral mutations that escape antibodies used to treat COVID-19. Science. 2021;371:850-854. doi:10.1126/science.abf9302

465. Starr TN, Greaney AJ, Hilton SK, et al. Deep mutational scanning of SARS-CoV-2 receptor binding domain reveals constraints on folding and ACE2 binding. Cell. 2020;182:1295-1310 e1220. doi:10.1016/j.cell.2020.08.012

466. Steed AL, Christophi GP, Kaiko GE, et al. The microbial metabolite desaminotyrosine protects from influenza through type I interferon. Science. 2017;357:498-502. doi:10.1126/science.aam5336

467. Stefan N, Birkenfeld AL, Schulze MB. Global pandemics interconnected—obesity, impaired metabolic health and COVID-19. Nat Rev Endocrinol. 2021;17:135-149. doi:10.1038/s41574-020-00462-1

468. Stefan N, Birkenfeld AL, Schulze MB, Ludwig DS. Obesity and impaired metabolic health in patients with COVID-19. Nat Rev Endocrinol. 2020;16:341-342. doi:10.1038/s41574-020-0364-6

469. Styer LM, Kent KA, Albright RG, Bennett CJ, Kramer LD, Bernard KA. Mosquitoes inoculate high doses of West Nile virus as they probe and feed on live hosts. PLoS Pathog. 2007;3:1262-1270. doi:10.1371/journal.ppat.0030132

470. Subbarao EK, London W, Murphy BR. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol. 1993;67:1761-1764. doi:10.1128/JVI.67.4.1761-1764.1993

471. Sugiura A, Kilbourne ED. Genetic studies of influenza viruses. 3. Production of plaque-type recombinants with A0 and A1 strains. Virology. 1966;29:84-91. doi:10.1016/0042-6822(66)90198-x

472. Sullivan C, Soos BL, Millard PJ, Kim CH, King BL. Modeling virus-induced inflammation in zebrafish: a balance between infection control and excessive inflammation. Front Immunol. 2021;12:636623. doi:10.3389/fimmu.2021.636623

473. Tai AW, Benita Y, Peng LF, et al. A functional genomic screen identifies cellular cofactors of hepatitis C virus replication. Cell Host Microbe. 2009;5:298-307. doi:10.1016/j.chom.2009.02.001

474. Takahashi T, Ellingson MK, Wong P, et al. Sex differences in immune responses that underlie COVID-19 disease outcomes. Nature. 2020;588:315-320. doi:10.1038/s41586-020-2700-3

475. Taniguchi T, Palmieri M, Weissmann C. A Qbeta DNA-containing hybrid plasmid giving rise to Qbeta phage formation in the bacterial host [proceedings]. Ann Microbiol (Paris). 1978;129B:535-536.

476. Teijaro JR, Ng C, Lee AM, et al. Persistent LCMV infection is controlled by blockade of type I interferon signaling. Science. 2013;340:207-211. doi:10.1126/science.1235214

477. Teo TH, Lum F-M, Claser C, et al. A pathogenic role for CD4+ T cells during Chikungunya virus infection in mice. J Immunol. 2013;190:259-269. doi:10.4049/jimmunol.1202177

478. Teo TH, Lum F-M, Ghaffar K, et al. Plasmodium co-infection protects against chikungunya virus-induced pathologies. Nat Commun. 2018;9:3905. doi:10.1038/s41467-018-06227-9

479. Tepasse PR, Hafezi W, Lutz M, et al. Persisting SARS-CoV-2 viraemia after rituximab therapy: two cases with fatal outcome and a review of the literature. Br J Haematol. 2020;190:185-188. doi:10.1111/bjh.16896

480. Tesla B, Demakovsky LR, Packiam HS, et al. Estimating the effects of variation in viremia on mosquito susceptibility, infectiousness, and

R0 of Zika in *Aedes aegypti*. PLoS Negl Trop Dis. 2018;12:e0006733. doi:10.1371/journal.pntd.0006733

481. Thangavel RR, Bouvier NM. Animal models for influenza virus pathogenesis, transmission, and immunology. J Immunol Methods. 2014;410:60-79. doi:10.1016/j.jim.2014.03.023

482. Thimme R, Wieland S, Steiger C, et al. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. J Virol. 2003;77:68-76. doi:10.1128/jvi.77.1.68-76.2003

483. Thompson BS, Moesker B, Smit JM, Wilschut J, Diamond MS, Fremont DH. A therapeutic antibody against west nile virus neutralizes infection by blocking fusion within endosomes. PLoS Pathog. 2009;5:e1000453. doi:10.1371/journal.ppat.1000453

484. Tracey LJ, An Y, Justice MJ. CyTOF: an emerging technology for single-cell proteomics in the mouse. Curr Protoc. 2021;1:e118. doi:10.1002/cpz1.118

485. Trobaugh DW, Gardner CL, Sun C, et al. RNA viruses can hijack vertebrate microRNAs to suppress innate immunity. Nature. 2014;506:245-248. doi:10.1038/nature12869

486. Trus I, Udenze D, Cox B, et al. Subclinical in utero Zika virus infection is associated with interferon alpha sequelae and sex-specific molecular brain pathology in asymptomatic porcine offspring. PLoS Pathog. 2019;15:e1008038. doi:10.1371/journal.ppat. 1008038

487. Tsatsanis C, Margioris AN, Kontoyiannis DP. Association between H1N1 infection severity and obesity-adiponectin as a potential etiologic factor. J Infect Dis. 2010;202:459-460. doi:10.1086/653842

488. Tumpey TM, Basler CF, Aguilar PV, et al. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science. 2005;310:77-80. doi:10.1126/science.1119392

489. Tumpey TM, García-Sastre A, Taubenberger JK, et al. Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. J Virol. 2005;79:14933-14944. doi:10.1128/JVI.79.23.14933-14944.2005

490. Tumpey TM, García-Sastre A, Taubenberger JK, Palese P, Swayne DE, Basler CF. Pathogenicity and immunogenicity of influenza viruses with genes from the 1918 pandemic virus. Proc Natl Acad Sci U S A. 2004;101:3166-3171. doi:10.1073/pnas.0308391100

491. Tyler KL, Mann MA, Fields BN, Virgin HW IV. Protective anti-reovirus monoclonal antibodies and their effects on viral pathogenesis. J Virol. 1993;67:3446-3453. doi:10.1128/JVI.67.6.3446-3453.1993

492. Tyler KL, McPhee DA, Fields BN. Distinct pathways of viral spread in the host determined by reovirus S1 gene segment. Science. 1986;233:770-774. doi:10.1126/science.3016895

493. Tyler KL, Virgin HW IV, Bassel-Duby R, Fields BN. Antibody inhibits defined stages in the pathogenesis of reovirus serotype 3 infection of the central nervous system. J Exp Med. 1989;170:887-900. doi:10.1084/jem.170.3.887

494. Uchiyama R, Chassaing B, Zhang B, Gewirtz AT. Antibiotic treatment suppresses rotavirus infection and enhances specific humoral immunity. J Infect Dis. 2014;210:171-182. doi:10.1093/infdis/jiu037

495. Uze G, Schreiber G, Piehler J, Pellegrini S. The receptor of the type I interferon family. Curr Top Microbiol Immunol. 2007;316:71-95. doi:10.1007/978-3-540-71329-6_5

496. Valenzuela DM, Murphy AJ, Frendewey D, et al. High-throughput engineering of the mouse genome coupled with high-resolution expression analysis. Nat Biotechnol. 2003;21:652-659. doi:10.1038/nbt822

497. Vandini S, Biagi C, Lanari M. Respiratory syncytial virus: the influence of serotype and genotype variability on clinical course of infection. Int J Mol Sci. 2017;18. doi:10.3390/ijms18081717

498. Varble A, Albrecht RA, Backes S, et al. Influenza A virus transmission bottlenecks are defined by infection route and recipient host. Cell Host Microbe. 2014;16:691-700. doi:10.1016/j.chom.2014.09.020

499. Vassilopoulos D, Calabrese LH. Rheumatic manifestations of hepatitis C infection. Curr Rheumatol Rep. 2003;5:200-204. doi:10.1007/s11926-003-0067-8

500. Vassilopoulos D, Calabrese LH. Extrahepatic immunological complications of hepatitis C virus infection. AIDS. 2005;19(Suppl 3):S123-S127. doi:10.1097/01.aids. 0000192080.56808.38

501. Venter JC, Sutton GG, Florea L, et al. The sequence of the human genome. Science. 2001;291:1304-1351. doi:10.1126/science.1058040

502. Ventura CV, Maia M, Travassos SB, et al. Risk factors associated with the ophthalmoscopic findings identified in infants with presumed zika virus congenital infection. JAMA Ophthalmol. 2016;134:912-918. doi:10.1001/jamaophthalmol.2016.1784

503. Vermillion MS, Ursin RL, Kuok DIT, et al. Production of amphiregulin and recovery from influenza is greater in males than females. Biol Sex Differ. 2018;9:24. doi:10.1186/s13293-018-0184-8

504. Vicens Q, Kieft JS. Shared properties and singularities of exoribonuclease-resistant RNAs in viruses. Comput Struct Biotechnol J. 2021;19:4373-4380. doi:10.1016/j.csbj.2021.07.024

505. Vignuzzi M, Stone JK, Arnold JJ, Cameron CE, Andino R. Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. Nature. 2006;439:344-348. doi:10.1038/nature04388

506. Virelizier JL, Hamet M, Ballet JJ, Reinert P, Griscelli C. Impaired defense against vaccinia in a child with T-lymphocyte deficiency

associated with inosine phosphorylase defect. J Pediatr. 1978;92:358-362. doi:10.1016/s0022-3476(78)80419-3

507. Virgin HW IV, Bassel-Duby R, Fields BN, Tyler KL. Antibody protects against lethal infection with the neurally spreading reovirus type 3 (Dearing). J Virol. 1988;62:4594-4604. doi:10.1128/JVI.62.12.4594-4604.1988

508. Virgin HW IV, Mann MA, Tyler KL. Protective antibodies inhibit reovirus internalization and uncoating by intracellular proteases. J Virol. 1994;68:6719-6729. doi:10.1128/JVI.68.10.6719-6729.1994

509. Virgin HW, Todd JA. Metagenomics and personalized medicine. Cell. 2011;147:44-56. doi:10.1016/j.cell.2011.09.009

510. Virgin HW, Walker BD. Immunology and the elusive AIDS vaccine. Nature. 2010;464:224-231. doi:10.1038/nature08898

511. Virgin HW, Wherry EJ, Ahmed R. Redefining chronic viral infection. Cell. 2009;138:30-50. doi:10.1016/j.cell.2009.06.036

512. Vischer HF, Vink C, Smit MJ. A viral conspiracy: hijacking the chemokine system through virally encoded pirated chemokine receptors. Curr Top Microbiol Immunol. 2006;303:121-154. doi:10.1007/978-3-540-33397-5_6

513. von Herrath MG, Dockter J, Oldstone MB. How virus induces a rapid or slow onset insulin-dependent diabetes mellitus in a transgenic model. Immunity. 1994;1:231-242. doi:10.1016/1074-7613(94)90101-5

514. von Herrath MG, Fujinami RS, Whitton JL. Microorganisms and autoimmunity: making the barren field fertile? Nat Rev Microbiol. 2003;1:151-157. doi:10.1038/ nrmicro754

515. Wahl A, De C, Fernandez MA, et al. Precision mouse models with expanded tropism for human pathogens. Nat Biotechnol. 2019;37:1163-1173. doi:10.1038/s41587-019- 0225-9

516. Wang F, Ma Y, Barrett JW, et al. Disruption of Erk-dependent type I interferon induction breaks the myxoma virus species barrier. Nat Immunol. 2004;5:1266-1274. doi:10.1038/ni1132

517. Wang P, Dai J, Bai F, et al. Matrix metalloproteinase 9 facilitates West Nile virus entry into the brain. J Virol. 2008;82:8978-8985. doi:10.1128/JVI.00314-08

518. Wang S, Ma P, Zhang S, et al. Fasting blood glucose at admission is an independent predictor for 28-day mortality in patients with COVID-19 without previous diagnosis of diabetes: a multi-centre retrospective study. Diabetologia. 2020;63:2102-2111. doi:10.1007/s00125-020-05209-1

519. Wang T, Rosen B, West AP, et al. Probability of phenotypically detectable protein damage by ENU-induced mutations in the Mutagenetix database. Nat Commun. 2018;9:441. doi:10.1038/s41467-017-02806-4

520. Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat Med. 2004;10:1366-1373. doi:10.1038/nm1140

521. Wang TT, Sewatanon J, Memoli MJ, et al. IgG antibodies to dengue enhanced for FcgammaRIIIA binding determine disease severity. Science. 2017;355:395-398. doi:10.1126/science.aai8128

522. Wang X, Li M, Zheng H, et al. Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. J Virol. 2000;74:11566-11573. doi:10.1128/jvi.74.24.11566-11573.2000

523. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet. 2009;10:57-63. doi:10.1038/nrg2484

524. Wei X, Decker JM, Wang S, et al. Antibody neutralization and escape by HIV-1. Nature. 2003;422:307-312. doi:10.1038/nature01470

525. Welsh RM, Che JW, Brehm MA, Selin LK. Heterologous immunity between viruses. Immunol Rev. 2010;235:244-266. doi:10.1111/j.0105-2896.2010.00897.x

526. Whelan SP, Ball LA, Barr JN, Wertz GT. Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. Proc Natl Acad Sci U S A. 1995;92:8388-8392. doi:10.1073/pnas.92.18.8388

527. Wieland S, Thimme R, Purcell RH, Chisari FV. Genomic analysis of the host response to hepatitis B virus infection. Proc Natl Acad Sci U S A. 2004;101:6669-6674. doi:10.1073/pnas.0401771101

528. Wieland SF, Chisari FV. Stealth and cunning: hepatitis B and hepatitis C viruses. J Virol. 2005;79:9369-9380. doi:10.1128/JVI.79.15.9369-9380.2005

529. Wilder-Smith A, Ooi EE, Horstick O, Wills B. Dengue. Lancet. 2019;393:350-363. doi:10.1016/S0140-6736(18)32560-1

530. Wilkinson TM, Li CKF, Chui CSC, et al. Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans. Nat Med. 2012;18:274-280. doi:10.1038/nm.2612

531. Winkler ES, Shrihari S, Hykes Jr BL, et al. The intestinal microbiome restricts alphavirus infection and dissemination through a bile acid-type I IFN signaling axis. Cell. 2020;182:901-918 e918. doi:10.1016/j.cell.2020.06.029

532. Won S, Eidenschenk C, Arnold CN, et al. Increased susceptibility to DNA virus infection in mice with a GCN2 mutation. J Virol. 2012;86:1802-1808. doi:10.1128/JVI.05660-11

533. Wong G, Qiu XG. Type I interferon receptor knockout mice as models for infection of highly pathogenic viruses with outbreak potential. Zool Res. 2018;39:3-14. doi:10.24272/j.issn.2095-8137.2017.052

534. Wong JP, Damania B. Modulation of oncogenic signaling networks by Kaposi's sarcoma-associated herpesvirus. Biol Chem. 2017;398:911-918. doi:10.1515/hsz-2017-0101

535. Wong K, Nguyen J, Blair L, et al. Pathogenesis of human immunodeficiency virus-mycobacterium tuberculosis co-infection. J Clin Med. 2020;9:3575. doi:10.3390/jcm9113575

536. Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic (H1N1) 2009 Influenza; Bautista E, Chotpitayasunondh T, Gao Z, et al. Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection. N Engl J Med. 2010;362:1708-1719. doi:10.1056/NEJMra1000449

537. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72314 cases from the Chinese Center for Disease Control and Prevention. JAMA. 2020;323:1239-1242. doi:10.1001/jama.2020.2648

538. Yamaoka S, Ebihara H. Pathogenicity and virulence of ebolaviruses with species- and variant-specificity. Virulence. 2021;12:885-901. doi:10.1080/21505594.2021.1898169

539. Yuan FF, Tanner J, Chan PKS, et al. Influence of FcgammaRIIA and MBL polymorphisms on severe acute respiratory syndrome. Tissue Antigens. 2005;66:291-296. doi:10.1111/j.1399-0039.2005.00476.x

540. Yuan L, Huang X-Y, Liu Z-Y, et al. A single mutation in the prM protein of Zika virus contributes to fetal microcephaly. Science. 2017;358:933-936. doi:10.1126/science.aam7120

541. Zerboni L, Reichelt M, Arvin A. Varicella-zoster virus neurotropism in SCID mouse-human dorsal root ganglia xenografts. Curr Top Microbiol Immunol. 2010;342:255-276. doi:10.1007/82_2009_8

542. Zhang Q, Bastard P, Liu Z, et al. Inborn errors of type I IFN immunity in patients with life-threatening COVID-19. Science. 2020;370:eabd4570. doi:10.1126/science.abd4570

543. Zhang R, Earnest JT, Kim AS, et al. Expression of the Mxra8 receptor promotes alphavirus infection and pathogenesis in mice and drosophila. Cell Rep. 2019;28:2647-2658 e2645. doi:10.1016/j.celrep.2019.07.105

544. Zhang R, Kim AS, Fox JM, et al. Mxra8 is a receptor for multiple arthritogenic alphaviruses. Nature. 2018;557:570-574. doi:10.1038/s41586-018-0121-3

545. Zhang SY, Jouanguy E, Ugolini S, et al. TLR3 deficiency in patients with herpes simplex encephalitis. Science. 2007;317:1522-1527. doi:10.1126/science.1139522

546. Zhao J, Li K, Wohlford-Lenane C, et al. Rapid generation of a mouse model for Middle East respiratory syndrome. Proc Natl Acad Sci U S A. 2014;111:4970-4975. doi:10.1073/pnas.1323279111

547. Zheng J, Roy Wong L-Y, Li K, et al. COVID-19 treatments and pathogenesis including anosmia in K18-hACE2 mice. Nature. 2021;589:603-607. doi:10.1038/s41586-020- 2943-z

548. Zhou H, Xu M, Huang Q, et al. Genome-scale RNAi screen for host factors required for HIV replication. Cell Host Microbe. 2008;4:495-504. doi:10.1016/j.chom.2008.10.004

549. Zinkernagel RM. Virus-induced immunopathology. In: Viral Pathogenesis. Lippincott-Raven; 1997:163-181.

550. Zinkernagel RM, Pfau CJ, Hengartner H, Althage A. Susceptibility to murine lymphocytic choriomeningitis maps to class I MHC genes—a model for MHC/disease associations. Nature. 1985;316:814-817. doi:10.1038/316814a0

551. Zuniga EI, Macal M, Lewis GM, Harker JA. Innate and adaptive immune regulation during chronic viral infections. Annu Rev Virol. 2015;2:573-597. doi:10.1146/annurev-virology-100114-055226

CHAPTER 9

Innate Immunity to Viruses

Akiko Iwasaki • John W. Schoggins • Sun Hur

Sensing viral infections

Viral sensing through pattern recognition receptors

Cell-autonomous virus recognition

RIG-I–like receptors

NOD-like receptors

Cell-extrinsic virus recognition

Toll-like receptors

Innate antiviral cytokines

Interferons

Type I IFN overview

Type I IFN induction

Type I interferon signaling

Type II interferon

Type III interferons

Inflammatory cytokines

Interleukin-6

Interleukin-1-Beta and interleukin-18

Tumor necrosis factor-alpha

Interleukin-15

Cell-intrinsic antiviral defense mechanisms

Interferon-induced antiviral defense

Cell types involved in innate immune responses to virus infections

Tissue-resident innate immune cells that support antiviral defense

Recruited innate immune cells for antiviral defense

Viral evasion of innate immunity

Differences between evasion strategy mechanisms of DNA vs RNA viruses

Conclusion

The innate immune system provides a universal form of host protection from infectious pathogens. Viruses enter the host either through the mucosal surfaces or transmitted by blood-feeding arthropods. Consequently, there are natural intrinsic immune defense mechanisms that block invading viruses at the mucosal surfaces and in blood. Even before the virus can enter the host cell, it faces barriers at the entry surfaces. The cornified epithelia of the skin provide a formidable barrier against viruses. At the surfaces of respiratory, gastrointestinal, and genitourinary tracts, the virus must penetrate the mucus layer coating to reach host target cells. The mucus is an aqueous secretion containing inorganic salts, antimicrobial enzymes, immunoglobulins, and glycoproteins such as lactoferrin and mucins. Viruses caught inside the mucus layer can be destroyed by antimicrobial enzymes. Mucus also assists in the removal of trapped viruses through mucociliary clearance mechanisms.

If the viruses manage to attach to the host cell epithelia lining the mucosal membranes, they must utilize cell surface molecules as viral entry receptors to enter the cell. Upon successful entry, viruses will be detected by a variety of pattern recognition receptors (PRRs) that sense unique features of viruses, mostly through the recognition of viral nucleic acids. Because viruses can infect most cell types of the host organism, every cell is equipped to detect and initiate immune responses against viruses. A key set of molecules induced by signaling downstream of PRRs are interferons (IFNs) and cytokines. IFNs induce hundreds of genes, known as IFN-stimulated genes (ISGs), that act on multiple stages of the viral life cycle to stop the production of viruses and prevent the spread of viruses. At the same time, both IFNs and cytokines induce the production of

chemokines that attract circulating immune cells to the site of infection. Recruited cells further contribute to host defense, by fortifying innate antiviral defenses, and by inducing adaptive immune responses. One of the main host innate immune defense strategies against viral pathogens is the elimination of the infected cells. This can be achieved by the effects of IFNs that promote the death of infected cells, or with the help of cytotoxic lymphocytes. Many additional antiviral defense mechanisms are mediated by ISGs to interfere with viral entry, replication, gene expression, virion assembly, and exit from the infected cells.

This chapter describes innate immune responses against viruses, with a primary emphasis on humans and other mammals. While many other innate antiviral functions are preserved throughout evolution, such as RNAi and xenophagy, this chapter will focus mostly on interferon and cytokine-induced antiviral defense mechanisms. It describes various host sensors that detect DNA and RNA viruses, signaling downstream of PRRs leading to IFN production. We will describe how these IFNs trigger the synthesis of ISGs and how they mediate their effector functions to provide an antiviral defense. We will describe viral evasion mechanisms against various host defense mechanisms. Finally, key cell types that are specialized to respond to viral infections are discussed.

Sensing Viral Infections

Viral Sensing Through Pattern Recognition Receptors

The mammalian immune system detects the presence of viruses through multiple mechanisms. The best-understood mechanisms involve various PRRs. The most common molecular patterns associated with virus infection are the features associated with viral nucleic acids. Innate immune recognition can be cell-intrinsic or cell-extrinsic, depending on whether it is mediated by infected or noninfected cells. Cell-intrinsic innate immune recognition is mediated by cytosolic sensors, including the RIG-I–like receptors (RLRs), cGAS, and NOD-like receptors (NLRs). Activation of these receptors generally occurs in infected cells. Accordingly, PRRs involved in cell-intrinsic recognition are broadly expressed because viral pathogens target a variety of cell types for replication. In contrast, cell-extrinsic innate immune recognition is mediated by transmembrane receptors, including Toll-like receptors (TLRs) and C-type lectin receptors (CLRs); their activation does not require the cells expressing these receptors to be infected. Cell-extrinsic recognition is mainly mediated by specialized cells of the immune system, such as macrophages and dendritic cells (DCs).

PRRs involved in microbial recognition have modular structures and share several structurally related domains for innate recognition, multimerization, interaction with adaptor molecules, and signaling (Fig. 9.1).

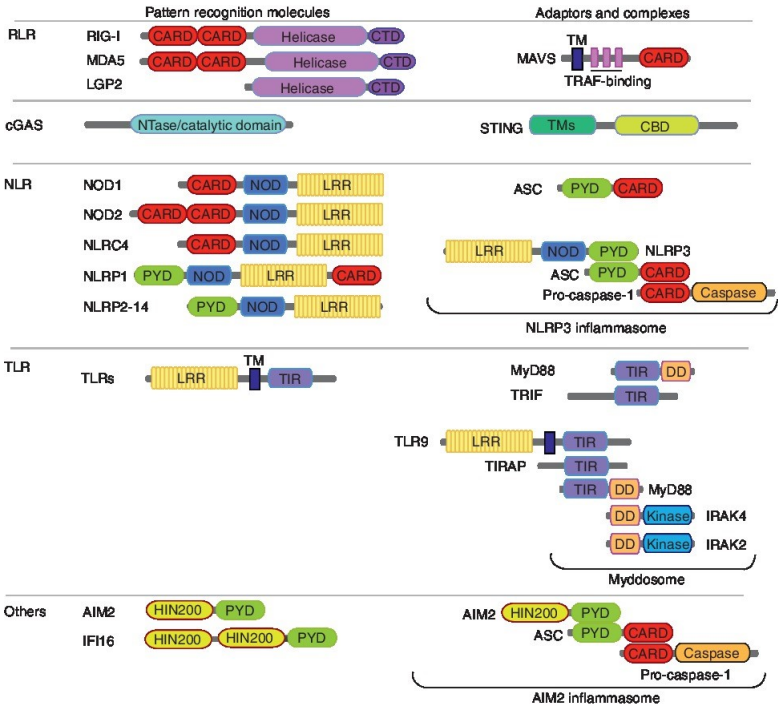

FIGURE 9.1 **Domain structure of viral sensors and adaptor molecules**. Pattern recognition molecules involved in viral sensing contain distinct domains, some of which are shared by different classes of receptors. These domains include CARD, caspase activation, and recruitment domain; CTD, C-terminal domain; DD, death domain; HIN200, hemopoietic IFN-inducible nuclear proteins is a 200-amino acid motif; LRR, leucine-rich repeat; NOD, nucleotide-binding oligomerization domain; NTase, nucleotidyltransferase; CBD, cGAMP-binding domain; PYD, pyrin domain; caspase domain; TIR, Toll/interleukin-1 receptor; TM, transmembrane region. Many of these domains can be used for homotypic oligomerization. Examples of molecular complexes are depicted for NLRP3 and AIM2 inflammasomes.

Cell-Autonomous Virus Recognition

RIG-I–Like Receptors

RNA viruses are recognized by the cytoplasmic RNA helicases, retinoic acid–inducible gene-I (RIG-I), and melanoma differentiation–associated gene 5 (MDA5).[246] The RLRs are expressed by most cell types. Both RIG-I and MDA5 recognize viral RNA through their helicase domains and the C-terminal domains (CTDs). Binding to viral RNA induces a series of conformational changes that include oligomerization of the helicase

domain and a caspase activation and recruitment domain (CARD) to initiate antiviral signaling[195,247] (Fig. 9.1). Both RIG-I and MDA5 utilize a common adaptor molecule called mitochondria antiviral signaling protein (MAVS),[207] which localizes to the mitochondrial membrane[207] and the peroxisomes.[59] MAVS directly recruits multiple TRAF E3 ligases (TRAF2, 3, 5, and 6) and activates TBK1-IKKi and IKKα/IKKβ/IKKg complexes, leading to activation and nuclear translocation of transcription factors IRF3 and NF-kB, respectively[62,136,211] (Fig. 9.2). MAVS can also activate IRF3 and NF-kB through mechanisms involving FADD-caspase-8/10,[222] TANK-NAP1,[196] and MEKK1.[248]

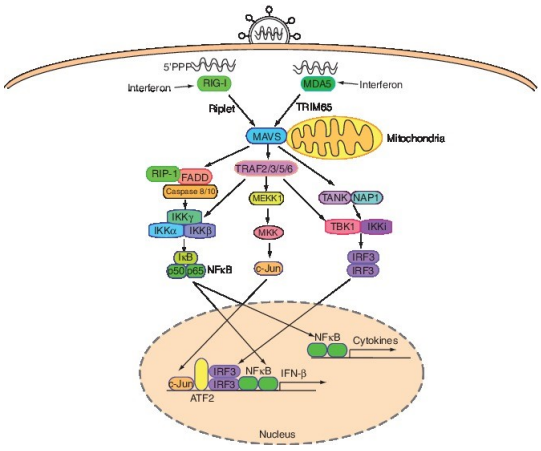

FIGURE 9.2 **RIG-I and MDA5 virus recognition pathways**. In a virally infected cell, RNA structures that are unique to viruses are recognized by RIG-I and MDA5. RIG-I detects dsRNA containing 5′ triphosphate, while MDA5 recognizes a long stretch of dsRNA. RNA binding to RLRs induces conformational changes, enabling their binding to MAVS adaptor protein expressed on the surface of mitochondria and peroxisomes. MAVS activates signaling to activate NF-kB, MAP kinases, and IRF3, leading to target gene expression including IFN-b and cytokines. Type I IFNs increase the levels of RLRs in bystander cells, enabling robust antiviral signaling upon infection.

RIG-I

RIG-I consists of two N-terminal CARDs, a central DExH box RNA helicase/adenosine triphosphatase (ATPase) domain and a zinc-binding CTD (Fig. 9.1). RIG-I functions as a critical PRR for many viruses including Sendai virus (SeV), vesicular stomatitis virus (VSV), influenza, hepatitis C virus, Japanese encephalitis virus, and Epstein-Barr virus.[110] RIG-I recognizes double-stranded RNA (dsRNA) that contains 5′-triphosphate or 5′-diphosphate ends, but not 5′-monophosphate, 5′-OH, or a 2′*O*-methyl group (as in the cellular mRNA cap structure).[78,187,199,204] While short (20 bp) dsRNA can bind and activate RIG-I when the duplex structure resides within a stem-loop,[118] duplex length >30-40 bp is generally optimal for robust activation of the RIG-I signaling.[32,177] RIG-I has an ATP-powered dsRNA translocation activity,[167] which enables its assembly into filamentous oligomers along the length of the dsRNA.[177,181] RIG-I oligomers then recruit the essential E3 ligase Riplet in an avidity-dependent manner (Fig. 9.2), allowing only the oligomeric RIG-I to be conjugated with K63-linked ubiquitin chains (K63-Ub_n).[32] In the presence of K63-Ub_n, RIG-I CARD domains from adjacent subunits form a homotetramer, serving as a seed to nucleate filament formation of MAVS.[180,243,249] MAVS filament functions as a signaling platform to recruit and activate further downstream signaling molecules.[90,137] This model suggests that RIG-I utilizes two distinct features of viral RNA simultaneously, the chemical structure of the 5′ end and duplex length. This mode of recognition can explain why RIG-I is more potently activated by SeV defective interfering (DI) particles than by wild-type virus.[20,216] However, for many other viruses, such as the influenza A virus, RIG-I ligands remain highly controversial and require future investigation.

MDA5

MDA5 senses many positive-strand viruses, such as picornaviruses and coronaviruses, and can be activated by cytosolic synthetic dsRNA, such as polyinosinic-polycytidylic acid (poly I:C).[75,110] There appears to be some level of redundancy in RIG-I and MDA5 in recognition of certain viruses. Dengue virus and West Nile virus are recognized by both MDA5 and RIG-I, and either of these sensors alone can induce type I IFN production.[144] Comparison of synthetic and viral dsRNA of various lengths revealed that MDA5 preferentially recognizes longer dsRNA that is at least ~1-2 kbp in length.[109] But this length threshold is not absolute—rather, it is dependent on dsRNA and MDA5 concentrations and other factors that together determine the stability of the MDA5 multimeric assemblies on dsRNA. MDA5 cooperatively assembles filaments along the length of the dsRNA.[22,179] MDA5 filament then recruits the essential E3 ligase TRIM65 (Fig. 9.2), which conjugates MDA5 with K63-Ub_n and activates CARD in a manner analogous to how Riplet activates RIG-I.[103,111] Unlike RIG-I, MDA5 filament formation does not require a triphosphate group at dsRNA 5′-end. Also unlike RIG-I, MDA5 does not translocate along dsRNA and does not require ATP hydrolysis to assemble filaments. These differences in assembly and disassembly mechanisms likely explain their distinct viral RNA specificity.

Studies also showed that both MDA5 and RIG-I can be aberrantly activated in the absence of viral infection, causing a spectrum of inflammatory disorders. Certain gain of function mutations in these receptors or loss of function mutations in their regulators (such as an RNA-modifying enzyme, ADAR1) lead to misrecognition of cellular RNAs by MDA5 and RIG-I and constitutive activation of the antiviral signaling pathways.[4,65,101,189,190] Environmental stress, such as certain chemotherapeutic agents or ionizing radiation, can also activate MDA5 and RIG-I through induction of virus-like cellular RNAs.[40,63,185,193]

LGP2

The third member of the RLR family, LGP2, harbors the helicase domain and CTD that are highly homologous to those of RIG-I and MDA5. However, LGP2 lacks a CARD and is unable to directly activate MAVS and downstream antiviral signaling pathways. LGP2 plays seemingly

complex regulatory roles for RLR signaling; LGP2 can either suppress or enhance RIG-I signaling depending on the context, while LGP2 enhances MDA5 signaling in response to both poly I:C and EMCV infection.[41,198,238] At least two distinct mechanisms have been proposed for how LGP2 stimulates MDA5 signaling. First, LGP2 was found to assist MDA5 filament nucleation, which is the rate-limiting step of MDA5 filament formation, thereby assisting the MDA5 signaling.[30] Second, LGP2 was shown to suppress Dicer's ability to process long viral dsRNA into siRNAs, thereby preserving MDA5 ligands and increasing the MDA5 signaling potential.[236,254] On the other hand, LGP2 was also shown to bind and sequester Ubc13, a subunit of the primary E2 conjugating enzyme for K63-Ub_n synthesis in the cytoplasm.[129] Considering both RIG-I and MDA5 signaling require K63-Ub_n, this could potentially function as a mechanism for their negative regulation. These multiple activities of LGP2 that can both positively and negatively affect different steps of the RLRs signaling pathway may explain the context-dependent effect of LGP2.

cGAS

cGAS is a sensor for cytoplasmic DNA. Upon double-stranded DNA (dsDNA) binding, cGAS undergoes a conformational change in the nucleotidyltransferase domain and joins ATP and GTP to synthesize cyclic GMP-AMP adduct (cGAMP).[3,58,72,218,252] cGAMP is then released from cGAS and functions as a second messenger to activate the downstream adaptor molecule STING, which is located on endoplasmic reticulum (ER) membranes[97] (Fig. 9.3). cGAMP binding triggers a conformational change in STING and induces STING oligomerization,[60,208,250,257] which enables recruitment and activation of TBK1 and IRF3 (Fig. 9.3). STING activation is accompanied by translocation of STING from ER to Golgi via the ER-Golgi intermediate compartment (ERGIC)[81] (Fig. 9.3). STING's redistribution to the ERGIC/Golgi compartment is required for STING signaling. While cGAS can be located in both the cytoplasm and the nucleus, only cytoplasmic cGAS is active because nuclear cGAS is tightly bound by the nucleosome in the inactive conformation, which prevents cGAS activation by the cellular DNA.[26,33,178,240,258] Cytoplasmic cGAS recognizes foreign DNA in a manner independent of DNA sequence, but dependent on double-stranded DNA (dsDNA) structure and length.[147] DNA length recognition is mediated by the cooperative assembly of ladder-like cGAS oligomers along DNA length.[9,132,253]

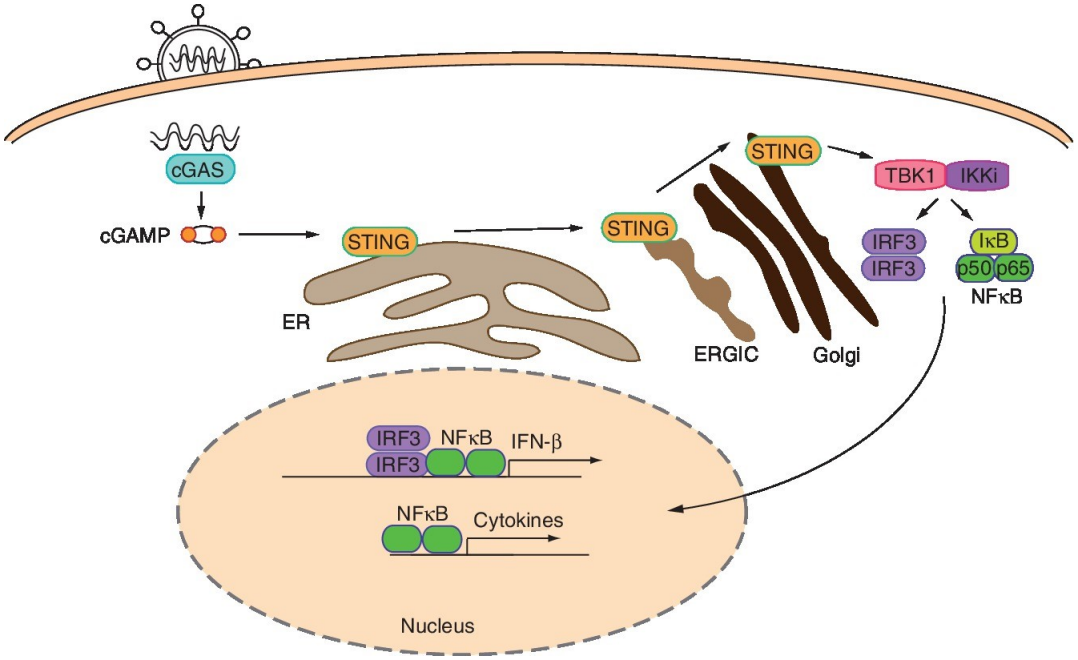

FIGURE 9.3 **cGAS virus recognition pathway**. Cytoplasmic DNA is recognized by cGAS. Upon dsDNA binding, cGAS joins ATP and GTP to synthesize cGAMP. cGAMP is then released from cGAS and activates STING localized on the ER membrane. cGAMP binding induces conformational changes in STING, enabling recruitment of TBK1 and activation of NF-kB and IRF3 to induce transcriptional activation of type I IFNs and proinflammatory cytokines. STING activation is coupled to and dependent on its translocation from ER to Golgi via the ERGIC intermediate.

Similar to RLRs, cGAS-STING signaling pathways can be activated in the absence of infection. For example, deficiencies in enzymes responsible for turning over endogenous DNA (eg, TREX1 and SAMHD1) can lead to autoimmune diseases due to the accumulation of cytosolic DNA and activation of the cGAS.[49,188,214] Gain-of-function mutations in STING also can result in aberrant activation of antiviral signaling and pathogenesis of inflammatory diseases.[102,140]

In addition to cGAS, other sensors can recognize viral DNA and activate antiviral innate immune responses. These include IFI16, DAI, and RIG-I (through Pol III–mediated RNA synthesis), which can play important roles depending on the DNA location and sequence composition.[2,43,223,234]

NOD-Like Receptors

NOD-like receptors (NLRs) comprise a large family of intracellular PRRs that regulate innate immunity in response to various pathogen infection and environmental stress.[155] The NLR family consists of multidomain proteins that contain a C-terminal LRR domain, a central NOD domain (a.k.a. NACHT domain), and an N-terminal effector domain. NLR proteins can be subdivided into three subfamilies depending on the structure of N-terminal domains—CARD-containing subfamily (NOD1, NOD2, NLRCs, CIITA), pyrin domain (PYD)-containing subfamily

(NLRPs) (Fig. 9.1), and BIR domain–containing subgroup (NAIPs) (not shown). Both NOD and NLRP members contribute to antiviral defense. The PYD subfamily of NLRPs consists of 14 members. Although the functions of many of the NLRPs are largely unknown, several NLRPs play a key role in the activation of caspase-1 by forming a multiprotein complex known as the "inflammasome" using the adaptor protein ASC[154] (Fig. 9.4). Caspase-1 is an essential mediator of the inflammatory response through its capacity to cleave and generate active forms of IL-1β and IL-18. IL-1β and IL-18 are potent proinflammatory cytokines. Activated caspase-1 also cleaves the pore-forming protein Gasdermin D, which releases the C-terminal repressor domain from the N-terminal pore domain and allows the formation of the multimeric pores in the plasma membrane[139,210] (Fig. 9.4). Gasdermin D pore facilitates cytokine release and induces an inflammatory form of cell death, namely pyroptosis. Formation and secretion of mature IL-1β and IL-18 require a two-step activation mechanism: first, transcriptional and translational upregulation of the pro-forms of these cytokines are induced by TLR and other cytokine stimulation (signal 1, priming), and a second signal that leads to the assembly of inflammasome and proteolytic activation of caspase-1 (signal 2, activation) (Fig. 9.4). Inflammasome complexes that are important in antiviral defense, in particular those activated by viral nucleic acid, are described below.

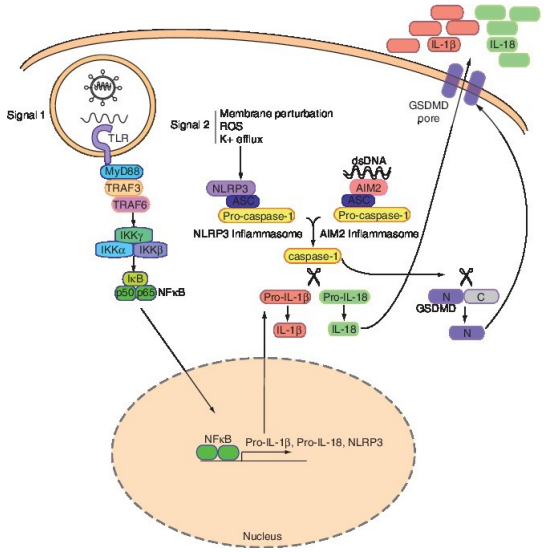

FIGURE 9.4 **Inflammasome activation by virus infection**. Virus infection can lead to inflammasome activation through two separate pathways. NLRP3 inflammasome is activated upon infection by adenovirus or influenza virus. This pathway is initiated by membrane perturbation, K^+ efflux, and reactive oxygen species (ROS). The second pathway involves AIM2, which is activated by dsDNA in the cytosol. Both NLRP3 and AIM2 form complex with ASC and pro-caspase-1 (inflammasome), leading to the self-cleavage and activation of caspase-1. Pro-forms of IL-1bβ and IL-18 induced by TLR signals are cleaved by activated caspase-1. Gasdermin D (GSDMD) is also cleaved, resulting in the formation of Gasdermin D pores in the plasma membrane. Mature forms of IL-1β and IL-18 are then released to the extracellular space through the Gasdermin D pore.

NLRP3-ASC Inflammasome

NLRP3 forms an ASC-dependent inflammasome (Fig. 9.4).[230] In the resting state, NLRP3 forms a cage-like assembly that is pre-localized on membranes within the trans-Golgi network.[8] The cage structure shields the PYD domains from activating ASC, but at the same time is a necessary state for activation. Upon stimulation, NLRP3 relocalizes to the microtubule-organizing center (MTOC) and engages with NEK7 kinase, which then triggers the assembly of the active disc-like complex.[209] This assembly stimulates NLRP3 PYD oligomerization, which seeds nucleation of the ASC filament through the PYD-PYD interaction and ASC CARD oligomerization. Oligomerized ASC CARD in turn recruits caspase-1 through the CARD-CARD interaction, which induces the oligomerization and activation of caspase-1.[145]

The NLRP3 inflammasome can be activated by a variety of stimuli including endogenous signals from dying cells (uric acid), crystals (asbestos, silica, alum) as well as microbial signals such as whole bacteria, bacterial RNA, pore-forming toxins, and fungal infection.[155] It is unclear whether microbial ligands can directly activate the NLRP3 inflammasome. Instead, the NLRP3 inflammasomes may sense signs of cellular damage, such as disruption in membrane integrity (through detection of K^+ efflux),[165,182] extracellular ATP released from stressed or damaged cells,[153] or disruption of mitochondria or trans-Golgi structure.[38,261] Virus infection also results in the activation of inflammasomes. Both SeV and influenza virus activate the NLRP3 inflammasome.[7,107,229] DNA viruses such as adenovirus also stimulate the NLRP3 inflammasome.[166] However, NLRP3 inflammasome is not activated by transfection of naked viral RNA, poly I:C, or infection with reovirus (dsRNA virus) or VSV (ssRNA virus),[115,166] indicating that viral RNAs are insufficient to trigger inflammasome activation. In the case of the influenza A virus, the NLRP3 inflammasome can be activated through the activity of the M2 ion channel[95] or the viral RNA sensor ZBP1.[124] Note that ZBP1 can also function independently of the NLRP3 inflammasome in a RIP1/3- and MLKL-mediated manner upon infection with influenza virus.[260] *In vivo*, *Nlrp3* deficiency resulted in increased susceptibility to high-dose influenza virus challenge.[7,229] In addition, after a sublethal dose of influenza virus, ASC-dependent inflammasome activation is required to elicit adaptive protective immunity to influenza virus, indicating the importance of NLRs in linking innate viral recognition to adaptive immunity.[94]

AIM2-ASC Inflammasome

Not all inflammasomes are activated by NLRPs. Recent studies showed that AIM2 couples cytosolic dsDNA recognition to ASC-caspase-1 inflammasome.[31,64,88,192] AIM2 is a HIN200 family of proteins that contains a dsDNA binding domain (HIN domain) and the PYD domain, which promotes interaction with the PYD domain of ASC (Fig. 9.1). AIM2 recognizes dsDNA in the cytosol and forms a filament-like structure along the length of dsDNA, detecting both dsDNA structure and length as key viral signatures.[104,163] Filamentation of the AIM2 HIN domain is

accompanied by oligomerization of AIM2 PYD, which then seeds ASC PYD filament[156] and in turn caspase-1 filament, leading to activation of caspase-1 (Fig. 9.4). Vaccinia virus, which contains a dsDNA genome, was shown to require AIM2 for recognition and activation of caspase-1 inflammasomes.[88] It is interesting to note that different classes of dsDNA viruses are recognized by NLRP3 (adenovirus) or AIM2 (vaccinia virus) for inflammasome activation. This likely depends on both the accessibility of the dsDNA genome to intracellular sensors, as well as the viral-induced cellular stress responses. While cytosolic dsDNA can bind AIM2 directly and activate inflammasomes, how and where exactly NLRP3 becomes activated by dsDNA viruses is yet to be determined.

IFI16

IFI16 is another DNA sensor that contains PYD and HIN domains. Similar to AIM2, IFI16 also forms filaments on DNA in a sequence-independent manner.[164] Unlike AIM2, however, IFI16 contains a nuclear localization signal and can function in both the nucleus and the cytoplasm, depending on the cell type and viruses.[131,237] IFI16 was shown to detect viral DNA within the nucleus and suppress its transcription by recruiting epigenetic silencing factors, such as PML, Sp100, and ATRX.[158,159] In the nucleus, IFI16 forms filaments on DNA to measure the length of the exposed stretch of DNA and to discriminate between chromatinized host genome vs naked viral DNA. Consistent with this idea, IFI16 restricts gene expression from the naked transfected SV40 DNA but not from prechromatinized SV40 DNA introduced into cells by viral infection.[174] Accordingly, viruses with unchromatinized DNA, such as HSV-1, can be detected by IFI16, which then promotes loading of nucleosomes and the addition of heterochromatin marks for silencing of viral genes.[174] IFI16 can also induce cytosolic innate immune signaling pathways, for example, by activating ASC inflammasomes, presumably through a mechanism similar to that of AIM2.[114] IFI16 was also reported to stimulate a type I interferon signaling pathway through STING.[175,234] It remains unclear whether IFI16 is exported to the cytoplasm and directly activates cytoplasmic STING and ASC.

Cell-Extrinsic Virus Recognition

Toll-Like Receptors

Toll-like receptors (TLRs) are one of the first mammalian PRR families to be characterized in depth. There are 11 functional TLRs in mice (TLR1-7, 9, 11-13) and 10 functional TLRs in humans (TLR1-10). All TLRs are type I transmembrane proteins with ectodomains containing leucine-rich repeat (LRR) that mediate the recognition of PAMPs. Some TLRs recognize bacterial, fungal, and protozoan pathogens, while others are dedicated to the viral recognition.[225] This chapter will focus on the TLRs that recognize viral pathogens.

The transmembrane domains of TLRs dictate the localization of the receptor, and the cytosolic domain contains a Toll/interleukin-1 receptor (TIR) domain, which binds to adaptor proteins and ultimately leads to the expression of a variety of genes.[5] TLR activation by viruses triggers a complex signaling cascade that bifurcates into two main pathways, culminating in the synthesis of proinflammatory cytokines (NF-kB dependent) and antiviral cytokines, type I interferons (IFNs) (IRF dependent). In addition, TLR signaling results in the activation of the mitogen-activated protein kinases (MAPKs) (Fig. 9.5). TLR recognition by DCs, the most potent antigen-presenting cells, enables their activation and antigen-presenting function, effectively linking innate recognition to the induction of adaptive immunity.[100]

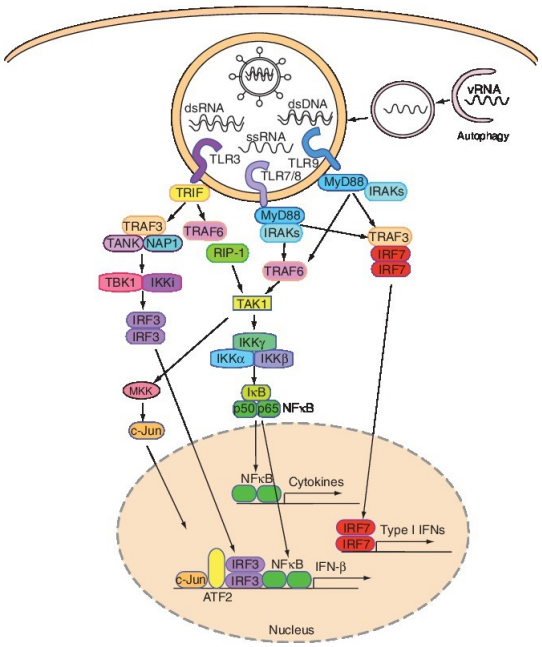

FIGURE 9.5 **Endosomal TLR virus recognition and signaling**. Viruses or virus-infected cells are endocytosed, and signatures of virus infection become accessible to TLRs upon digestion of membranes and nucleocapsid. TLR3 recognizes dsRNA, TLR7 and TLR8 recognize ssRNA, and TLR9 recognizes DNA. In addition, autophagy delivers TLR7 ligand (cytosolic viral replication intermediate) by fusion with the endosomes containing TLR7. Proteolytically activated TLRs in the endosomes recruit adaptor proteins and induce downstream signaling to activate a variety of transcription factors. IRF3 in combination with NF-kB, c-Jun, and ATF bind to the promoter and induce IFN-b expression. IRF7 homodimer binds to promoter regions of IFN-a genes and induces IFN-a expression. In addition, NF-kB activates transcription of a large number of genes including cytokines.

TLRs are distributed on specific subcellular locations and are specifically expressed by different cell types. TLRs can be categorized into two

major types, those that are expressed on the cell surface (TLR1, 2, 4, 5, 6, 11, 12) and those that are expressed in the endolysosomes (TLR3, 7, 8, 9). Although virus-TLR interaction can occur with both types of TLRs, many of the virus interactions with surface TLRs represent a viral invasion mechanism (beneficial to the virus) rather than PAMP recognition by TLRs (beneficial to the host). In this section, we will discuss the latter type of TLR-virus PAMP recognition leading to antiviral defense.

Viruses are recognized by a group of TLRs that reside in the endosomal membrane, namely, TLR3, 7, 8, and 9, where they can gain access to viral nucleic acids upon endocytosis of virions or nucleic acids released from infected and dead cells. A major advantage of this strategy of viral recognition is that the viral sensing and subsequent induction of type I IFN genes can occur in uninfected cells (thus called cell-extrinsic sensing) and, therefore, are not subjected to viral evasion mechanisms. In addition, since all viruses contain a genome consisting of RNA or DNA, such molecular patterns can be surveyed by a very limited number of receptors. These endosomal TLRs require trafficking by the endoplasmic reticulum (ER) membrane protein, UNC93B1,[29,221] and further regulation by UNC93B1.[150,151] Mutations in the *Unc93b1* gene were found in two patients with severe HSV encephalitis,[34] indicating the importance of transport of endosomal TLRs via UNC93B1 in antiviral defense. Within the endosome, TLR3, 7, and 9 additionally undergo processing by endolysosomal proteases cathepsins, which is required for receptor activation and downstream signaling.[61,108,176,232] Dependence of TLRs activation upon proteolytic processing in the endolysosomes provides an additional mechanism to ensure that aberrant activation of TLRs from the cell surface or other cellular location by self ligands is avoided.

TLR3

TLR3 is expressed by a variety of cells in humans and mice, including conventional DCs, B cells, fibroblasts, and epithelial cells but not in plasmacytoid DCs (pDCs). Upon PAMP recognition, TLR3 recruits TRIF via its TIR domain (Fig. 9.5). TRIF activates both NF-kB and IRF3, leading to proinflammatory cytokines and type I IFN gene expression, respectively. TLR3 recognizes dsRNA that is often generated during replication of RNA viruses (except for retroviruses).[6] DNA viruses also produce dsRNA by convergent transcription of their genomes.[241] TLR3 responds to the artificial dsRNA mimic, poly I:C, when it is provided extracellularly.[75,110] Structures of TLR3 in complex with dsRNA showed that TLR3 utilizes LRRs to recognize dsRNA structure,[130] binds dsRNA as a dimer, and possibly higher-order oligomer,[130,251] leading to a graduated response that increases with dsRNA length.[135] However, evidence supporting TLR3-mediated recognition of authentic viral replication intermediates remains elusive, possibly due to overlapping recognition pathways that can compensate for the loss of TLR3. TLR3 may also recognize virus-produced dsRNA in the context of phagocytized apoptotic cells.[205] Humans with dominant-negative mutations in the *TLR3* gene suffer from neonatal herpesvirus infection,[251] indicating the importance of this receptor in protection against HSV-1 in the CNS.

TLR7/8

In humans, TLR7 is expressed in plasmacytoid DCs and B cells, while TLR8 is expressed by myeloid DCs and monocytes. In mice, TLR7 is expressed by pDCs, conventional DCs (except for DC1s), and B cells. The function of the mouse TLR8 is still poorly understood. However, TLR8-deficient mice develop autoimmunity due to hyperstimulation of TLR7.[52] TLR7, 8, and 9 are all expressed in the endosomes and share similar signaling pathways. Upon cognate ligand engagement, these receptors recruit TIRAP via their TIR domains.[25] This interaction triggers oligomerization of TIRAP TIR domains, which in turn nucleates polymerization of MyD88 filament through TIR-TIR interactions.[45] The oligomer of MyD88 subsequently recruits IRAK2 and 4 through their death domains (DDs), resulting in the formation of a macromolecular assembly termed the myddosome.[133] Within the myddosome, IRAK2/4 triggers the downstream signaling cascades for activation of the NF-kB and IRF7 (Fig. 9.5).

TLR7 and TLR8 both induce innate immune responses to ssRNA-viruses. TLR7 signaling induces type I IFN and cytokine responses to influenza, SeV, and VSV.[56,148] While the influenza virus is recognized upon endocytosis by TLR7, other viruses such as VSV and SeV require replication in the cytosol before recognition by TLR7. These viruses are recognized by TLR7 in the endosome upon delivery of cytosolic viral replication intermediates through the process of autophagy.[128] Human TLR8 appears to function as a bacterial RNA sensor,[121] similar to the murine TLR13.[173] At the molecular level, TLR7 and TLR8 recognize degradation products of ssRNA.[227,255] Both TLR7 and TLR8 contain two distinct ligand-binding sites. Site 1, which is highly conserved between TLR7 and TLR8, recognizes nucleosides (G for TLR7 and U for TLR8). Unlike site 1, site 2 of TLR7 differs from that of TLR8, recognizing distinct degradation products of ssRNA. TLR7 site 2 binds successive U-containing ssRNA,[256] while TLR8 site 2 binds purine-2′3′-cyclophosphate generated by RNase T2-mediated cleavage.[80] Ligand binding at site 2 further stabilizes the ligand binding at site 1 and TLR dimerization.

TLR9

TLR9 is expressed in pDCs and B cells in humans. Mice express TLR9 in pDCs, B cells, macrophages, and conventional DCs. TLR9 is located in the endosome and mediates the recognition of viral DNA. Bacterial DNA sequences containing hypomethylated CpG motifs were shown to activate TLR9,[86] but the sugar-base-backbone, 2-deoxyribose, of phosphodiester DNA also plays an important role.[82] Crystal structures of TLR9 in complex with DNA showed that TLR9 binds ssDNA as a dimer.[170,171] As with TLR7/8, two sites are used to recognize both CpG DNA and 5′-TCG/TCC. Ligand engagement of both sites is important for TLR9 activation. Interestingly, a TLR9 molecule “retargeted” to the plasma membrane was unable to respond to viral nucleic acids, however, now responded to self DNA which did not stimulate wild-type TLR9.[19] These results suggest that not only is endosomal localization important to trigger TLR-viral nucleic acid interactions but that endosomal TLRs may limit access to non-viral nucleic acids via an active sequestering mechanism in the endosome.

C-Type Lectins

In addition to TLRs, CLRs expressed on the plasma membrane can bind to certain viruses. Classical CLRs contain a carbohydrate recognition domain responsible for the $Ca2^{+}$-dependent binding to their ligands. However, carbohydrate-recognition domains of many CLRs do not bind to $Ca2^{+}$.[12] CLRs can be classified into those that are used solely for endocytosis of pathogens, and those that are used for inducing signaling. The endocytic CLRs include mannose receptor, DEC205, and langerin, which are expressed on DC1s and Langerhans cells, respectively. Langerin on Langerhans cells is capable of clearing HIV by receptor-mediated endocytosis and degradation in Birbeck granules.[51]

The signaling CLRs include Dectin-1 and DC-SIGN.[53] Dectin-1, which is a receptor for fungal β-glucans, contains an ITAM motif in the cytosolic domain and engages Syk tyrosine kinase and CARD9, leading to NF-kB activation.[113] Dectin-1 plays an important role in antifungal defense, but it is not known to have antiviral functions. DC-SIGN binds to HIV-1 and Ebola virus. Signaling through DC-SIGN alone does not lead to the activation of NF-κB or expression of cytokines, but it can modulate signaling through other PRRs. DC-SIGN engagement by viruses leads to serine/threonine kinase Raf-1 activation. After translocation of NF-κB by TLR-stimulation, DC-SIGN–activated Raf-1 mediates the phosphorylation of NF-κB subunit p65, which in turn leads to p65-acetylation. Acetylation of p65 both prolongs and increases IL-10 transcription, resulting in increased IL-10 production. However, the majority of C-type lectin-virus interactions reflect the viral invasion mechanism. A clear example of this is DC-SIGN signaling by HIV-1. This interaction results in impaired DC maturation, enhanced T-cell proliferation, and transmission of HIV-1 to T cells, thereby promoting systemic infection of the host. DC-SIGN and a related C-type lectin, DC-SIGNR, also enhance infection by Ebola virus.[17] Another example is the dengue virus, which binds to CLEC5A and induces signaling through DAP12 to induce proinflammatory cytokines. This leads to lethality in infected mice.[39]

Innate Antiviral Cytokines

Interferons

Antiviral mechanisms in vertebrates are highly dependent on the action of interferons, a group of cytokines that are induced when a cell senses a viral infection. There are three types of interferons: type I (a, b, e, k, w, t), type II (g), and type III (l) (Fig. 9.6). The type I IFNs are the best studied for antiviral activity. Type III IFNs are also emerging as key players in innate immunity, primarily at mucosal surfaces, as described below. Type II IFN is often considered an antibacterial cytokine, though it also has bona fide antiviral properties. This section will focus primarily on the antiviral actions of type I and III IFNs, and type II IFN will be discussed below.

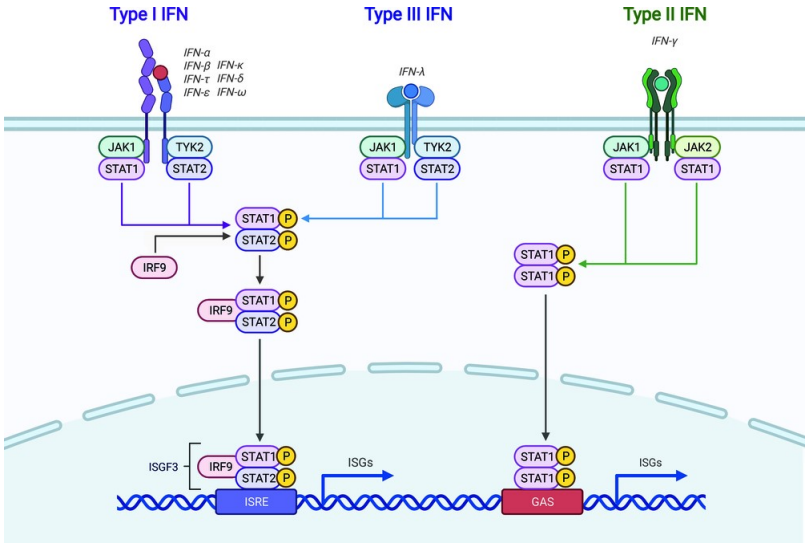

FIGURE 9.6 **IFN receptor signaling.** Type I, II, and III IFNs initiate signaling by binding their cognate receptors IFNAR, IFNGR, and IFNLR. Engagement of the receptors induces activation of prebound kinases (JAK1 and TYK2), leading to phosphorylation of STAT proteins. Phosphorylated STAT molecules form dimers, which translocate into the nucleus to initiate the transcription of genes. STAT1/STAT2 dimers recruit IRF9, forming ISGF3, which binds to ISRE sequence found in the promoter of many ISGs. STAT1 homodimer binds to the GAS sequence to induced transcription of a set of ISGs that partially overlaps with the ISGF3-dependent genes.

Type I IFN Overview

Type I IFNs are key cytokines for inducing an antiviral state in infected and uninfected neighboring cells,[96] and for promoting adaptive immune responses.[215] In humans, the type I IFNs consist of 13 α-genes coding for 12 IFN-α subtypes and single genes encoding IFN-β, IFN-ε, IFN-κ, and IFN-ω. IFN-β is a central cytokine in antiviral immune responses, as it can be produced by all nucleated cells upon infection. IFN-α subtypes are also critical in antiviral defenses, and many of the genes encoding IFN-α are directly induced by IFN-β signaling, resulting in amplification of the antiviral response. Other type I IFNs listed above have a tissue-specific expression (IFN-κ, IFN-ε), or they are expressed exclusively in certain lineages of mammals (IFN-τ, IFN-δ).

Type I IFN Induction

As described in detail above, stimulation of various PRRs including TLRs, RLRs, and cytosolic DNA sensors leads to the production of type I IFNs. Most virally infected cells trigger the cell-intrinsic pathway of type I IFN production through activation of RLRs or DNA sensors, which leads to phosphorylation of IRF3 and induction of IFN-β. Antigen-presenting cells additionally recognize viral genomes within the endosome via TLR7 or TLR9 and induce robust secretion of IFN-a and IFN-β. pDCs express high constitutive levels of IRF7 and are thought to be the only cells capable of coupling TLRs to IRF7 directly, thereby leading to rapid and robust transcription of type I IFN genes. IRF7 is also involved in amplifying the interferon response. The *IRF7* gene is induced by IFN-β, and IRF7 transcriptionally stimulates the production of additional IFN genes. Additionally, IFN signaling also increases the expression of some RLRs and TLRs, further augmenting the response.

Type I Interferon Signaling

The type I IFN receptor, herein referred to as IFNAR, is a heterodimeric receptor composed of chains encoded by IFNAR1 and IFNAR2 genes (Fig. 9.6). The canonical pathway for type I IFN signaling is depicted in Figure 9.6. Upon engagement of type I IFNs with the receptor, the receptor subunits dimerize resulting in phosphorylation of Tyk2, which is associated with the IFNAR1, by Janus kinase (JAK) 2. Activated Tyk2 subsequently phosphorylates JAK1, which is coupled to the IFNAR2 chain. Signal transducer and activator of transcription (STAT) 1 and 2 are prebound to the IFNAR2 chain. Activated JAK1 binds to STAT2 and phosphorylates it, creating a binding site for STAT1, and resulting in dimerization of STAT1 and STAT2. The STAT1-2 heterodimer forms a transcription complex, ISGF3, with IRF9 to facilitate transcription of ISGs by binding to sequences called interferon-simulated response elements (ISREs). In contrast to type I IFNs, type II IFN (IFN-g) signaling results in STAT1 homodimers that bind to the IFN-g–activated site (GAS) sequence motif (Fig. 9.6).

Type II Interferon

IFN-γ is secreted mainly by activated lymphocytes, such as T cells and NK cells, though other immune cells can also produce IFN-γ under certain conditions. The interferon-gamma receptor (IFNGR) is analogous to other IFN receptors and consists of two subunits: IFNGR1, the ligand-binding chain, and IFNGR2, the signal-transducing chain (Fig. 9.6). Receptor assembly leads to activation of JAK-STAT signaling, with STAT1 homodimers being the principal transcription factor that binds GAS elements in the promoters of responsive genes. IFN-γ is not as well known for its antiviral properties as other antiviral interferons, but it does exert antiviral activity by inducing the transcription of a set of ISGs that overlap with those induced by type I IFN.[55]

Type III Interferons

In humans, there are 4 type III IFN genes: *IFNL1*, *IFNL2*, *IFNL3*, and *IFNL4*. All have antiviral activity and loss of IFNL4 expression in certain human populations correlates with viral diseases, most notably hepatitis C.[184,228] Type III IFNs share many similarities with the type IFNs, though there are notable exceptions.[119,127,160,245] A major difference is that type III IFNs use a different receptor, IFNLR, which is a heterodimer IFNLR1 and IL-10Rb (Fig. 9.6). The intracellular JAK-STAT signaling cascade triggered by IFNL engagement with the receptor is mostly identical to the type I IFN–induced cascade, leading to the induction of similar sets of ISGs. In addition to distinct receptor utilization, IFNLR is also not as widely expressed as IFNAR. It is primarily expressed in mucosal tissues such as lung and intestinal epithelial cells. Thus, type III IFNs are emerging as key antiviral defense molecules at barrier surfaces. Type III IFNs are generally also less potent than type I IFNs, with slower signaling kinetics, and less induction of inflammatory signature due to the limited tissue distribution of the receptor.

Inflammatory Cytokines

In addition to type I IFNs, many inflammatory cytokines play an important role in antiviral defense, by either directly inducing antiviral effector molecules, or indirectly by stimulating cellular recruitment, phagocytosis of infected cells, and activating adaptive immune responses such as cytotoxic T lymphocytes and neutralizing antibodies. Cytokines can act locally through autocrine and paracrine mechanisms, or, if produced at high enough levels, they can gain access to the circulation and induce systemic effects, such as acute phase response or fever. The major inflammatory cytokines IL-6, IL-1β, and TNF-α are produced by virally infected DCs and macrophages and act systemically with a wide spectrum of biological activities that help to coordinate the body's response to infections. These cytokines are responsible for causing fever by inducing prostaglandin E2, which acts on the hypothalamus. This increases heat production from brown fat and induces vasoconstriction to prevent heat loss through the skin. Fever is thought to be generally beneficial to host defense, because some pathogens may replicate less efficiently at high temperatures. However, the role of fever in host defense is still incompletely understood. IL-6, IL-1β, and TNF-α also stimulate the acute phase response in the liver. Acute-phase proteins secreted by hepatocytes can have direct antiviral effects, for example, by promoting their killing by complement or phagocytes. Finally, IL-6, IL-1β, and TNF-α induce a leukocytosis, leading to an increase in circulating neutrophils, which are phagocytes that help to clear virally infected cells. When these inflammatory cytokines are overstimulated or chronically induced, however, they can lead to cytokine storm and immunopathology.

Interleukin-6

IL-6 is induced by virus infections downstream of most PRR signaling. IL-6 binds to IL-6R, which belongs to the hemopoietin family of receptors, which are tyrosine kinase–associated receptors that form dimers upon binding their cytokine ligand. IL-6R consists of IL-6Ra and gp130 chains. IL-6 bound to the IL-6Ra chain causes the association of the complex with gp130, resulting in the activation of Jak1, Jak2, and Tyk2, followed by the phosphorylation of Stat3. Phospho-Stat3 dimerizes and translocates into the nucleus, where it activates the transcription of target genes. IL-6 is a pleiotropic cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation, and oncogenesis.[117] IL-6 is induced by a variety of signals including TLR, NLR, and RLR signaling and is a key cytokine that triggers the acute phase response, as well as activation of T cells and antibody-producing plasma cells.

Interleukin-1-Beta and Interleukin-18

As described above, secretion of IL-1β and IL-18 requires the activation of inflammasomes. Both IL-1R and IL-18R are heterodimers of a ligand binding a chain and a coreceptor accessory protein (AcP). Both chains contain a TIR domain in the cytoplasmic tail. As such, these receptors recruit MyD88 and induce NF-kB signaling (Fig. 9.7). IL-1β through binding to IL-1R induces the expression of hundreds of genes, including cytokines (IL-6 and TNF-α), chemokines (eg, IL-8), and adhesion molecules that are important for the leukocyte trafficking.[57] In adaptive antiviral immunity, IL-1β plays an important role in the antigen-driven expansion and differentiation of CD4 T cells.[21] While IL-1R is widely expressed by many cell types, IL-1β is mainly produced by the sentinel cells of the innate immune system including macrophages and DCs, although fibroblasts and keratinocytes can also synthesize this cytokine in response to tissue injury or stress signals.[57,212] On the other hand, IL-18, which is expressed mainly by macrophages and DCs, works alone or in combination with IL-15 prime natural killer (NK) cells to produce IFN-γ and enhance their cytolytic activity.[67,92,226] IL-18 in combination with IL-12 or IL-2 can also induce differentiation of either Th1 or Th2 cell types depending on the cytokine milieu.[89,172] IL-18 is also required for optimal cytokine production by CTLs including IFN-γ, TNF-α, and IL-2.[54]

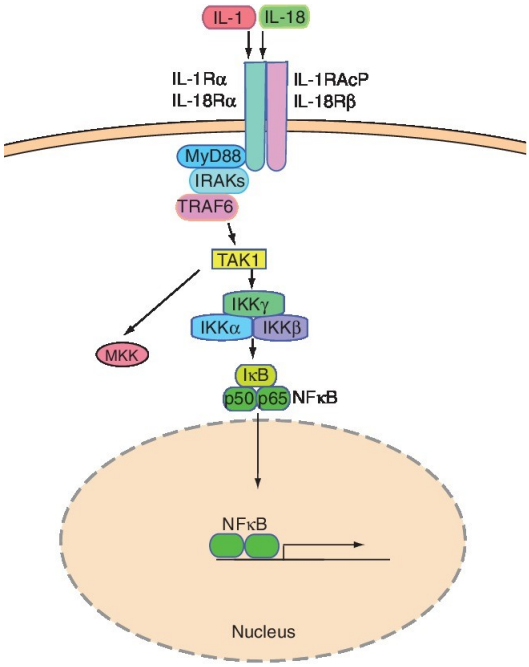

FIGURE 9.7 **IL-1R and IL-18R signaling**. IL-1R consists of the IL-1Ra chain and IL-1RAcP chain, while IL-18R consists of the IL-18Ra chain and IL-18Rb chain. Each chain contains a TIR domain in the cytoplasmic region, and upon receptor engagement by IL-1 or IL-18, recruits MyD88 and induces signaling resulting in NF-kB and MAPK activation.

IL-1β plays an integral role in antiviral immunity against influenza A virus infection. IL-1R$^{-/-}$ mice infected with influenza virus have impaired leukocyte recruitment to the lung, diminished CD4 and CD8 T-cell responses, impaired immunoglobulin responses, and succumb to virus-induced fatality.[94,200] On the other hand, mice deficient in IL-18 were found to have a transient increase in viral titer due to defective NK cytotoxicity after influenza A virus infection but otherwise had normal adaptive immunity to influenza infection.[134] Excessive levels of IL-1β and IL-18 produced during viral infection are associated with mortality from SARS-CoV-2.[146]

Tumor Necrosis Factor-Alpha

TNFR belongs to a group of receptors known as the TNFR family, characterized by a cysteine-rich common extracellular binding domain. Upon engagement of the ligand TNF, TNFR forms a trimer (Fig. 9.8). TNFRI contains a cytoplasmic death domain, which recruits the adaptor TRADD through its death domain. TRADD can assemble two different signaling complexes, involving FADD pro-caspase-8 or RIP1 and TRAF2. RIP1 activates IKK, resulting in the activation of NF-κB, while TRAF2 stimulates the JNK signaling pathway. Like IL-1β and IL-6, TNF is involved in the induction of a stereotypic inflammatory response that promotes host defense from a broad range of pathogens, including viruses. Recent studies revealed an additional pathway activated by TNFR—the induction of cell necrosis via the protein kinase RIP3. This TNF-induced necrosis was shown to be important for immune defense against vaccinia virus and CMV.[44,235] TNF blockers widely used to treat rheumatoid arthritis and other chronic inflammatory diseases have revealed the importance of TNF in the prevention of infection and reactivation of various viruses such as varicella-zoster virus, Epstein-Barr virus, cytomegalovirus, human papillomavirus, and JC virus.[116] However, the incidence of these infections in TNF blockade remains uncommon.

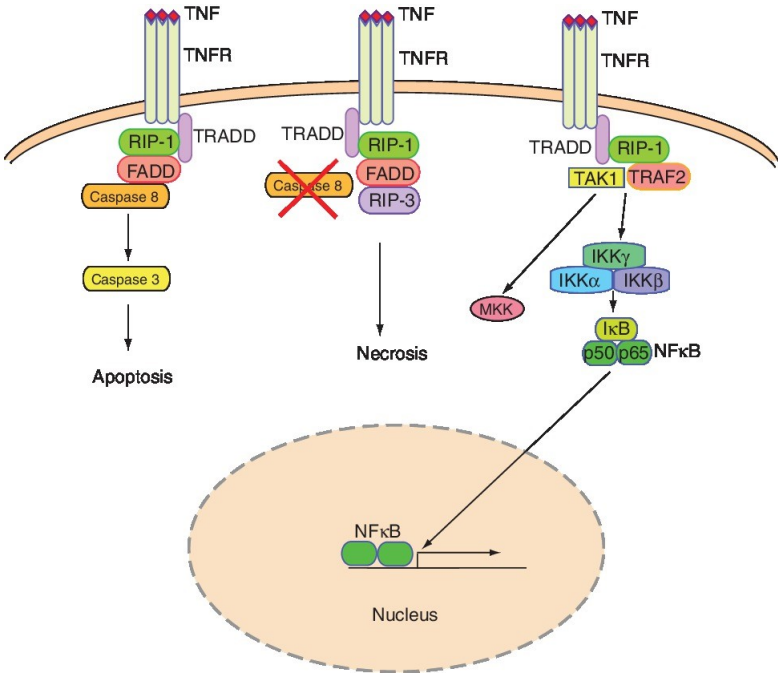

FIGURE 9.8 **TNFR signaling leads to three distinct cell fates**. TNF binding trimerizes the TNFR. Through the cytoplasmic death domain, TNFR recruits TRADD, which also contains a DD. TRADD can assemble different signaling complexes. TRADD can recruit FADD through DD-DD interaction, which results in caspase-8 activation, leading to apoptosis. In cells in which caspase-8 is rendered nonfunctional, RIP-3 is recruited to the complex to induce necrosis. In addition, TRADD can also recruit RIP-1 and TRAF2, resulting in signal transduction and activation of NF-kB and MAP kinase pathways.

Interleukin-15

IL-15 is produced by DCs and macrophages upon viral infection. IL-15R is a trimer of the IL-15Rα chain, which is specificto IL-15, the β chain, and the common γ chains that are shared with IL-2 and other cytokines. An unusual feature of the IL-15R complex is that the IL-15Rα chain is expressed on the cells that produce IL-15, rather than on the target cell that responds to IL-15. IL-15 forms a complex with the IL-15Rα chain in the ER and the two proteins are transported to the cell surface where they remain associated and function as a ligand for the IL-15Rβ and the common γ chains expressed on target cells. IL-15R induces activation of STAT5, resulting in the expression of antiapoptotic and growth-promoting genes in target cells.[152] The main target cell types of IL-15 are cytotoxic lymphocytes: IL-15 is an important growth and survival factor for CD8 T cells, NK cells, and a subset of NKT cells. Importantly, IL-15 can function downstream of type I IFNs in antiviral immune responses.[169]

Cell-Intrinsic Antiviral Defense Mechanisms

Interferon-Induced Antiviral Defense

IFN signaling leads to the induction of hundreds of ISGs, as demonstrated by many transcriptome profiling studies over the past 20+ years.[55] While a small fraction of the induced genes is noncoding, the vast majority encode specific proteins that together exert the antiviral, antiproliferative, and proinflammatory activities typical of an IFN response. Genetic screens have begun to catalog which ISGs encode antiviral effectors.[138,203] The picture emerging from many studies is that although IFN induces hundreds of ISGs, a relatively small fraction of them has antiviral activity. Indeed, IFN induces many proteins such as MHC molecules, cytokines, chemokines, and their receptors, all of which help stimulate adaptive immunity but do not have intrinsic antiviral activity.

Antiviral ISG effectors have specific mechanisms that usually target viruses at some stage of their replication cycle. Many reviews have covered this topic[37,201,202]; therefore, rather than present an exhaustive list of known antiviral effectors, general themes will be presented, with certain effectors mentioned as notable examples (Fig. 9.9).

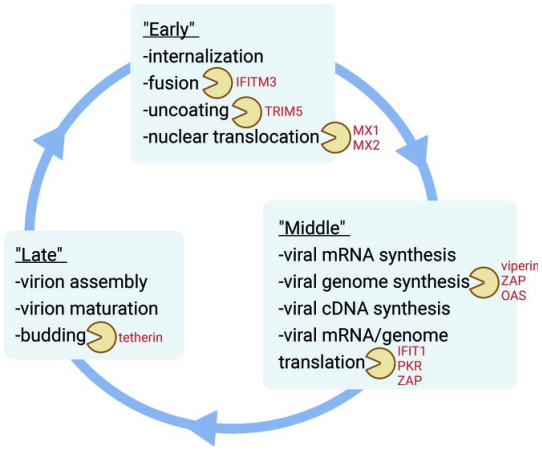

FIGURE 9.9 **Type I IFN–induced antiviral mechanisms**. Antiviral IFNs induce hundreds of genes, many of which encode effectors that establish an antiviral state. ISG-encoded effectors can target the viral replication cycle at any stage, including entry, translation, replication, and egress. Selected examples are depicted in the figure.

Inhibition of Viral Entry

Viruses must enter cells as a first step in the replication cycle, and this step is a key target of ISG-mediated defense. A family of ISGs that inhibits viral entry is the interferon-induced transmembrane (IFITM) protein family, encoded by genes *IFITM1*, *IFITM2*, and *IFITM3* as well as two other genes with no known roles in immunity. The best characterized of these is IFITM3, which suppresses infection of numerous viruses, including flaviviruses and influenza A virus. IFITM3 inhibits these endocytic viruses by preventing the fusion of their membranes with the endosomes.[27,259] This prevents the release of the genome into the cytosol, effectively halting replication.

In a unique mechanism of postentry inhibition, TRIM5α, an E3 ligase, forms a lattice on the surface of retrovirus capsids and mediates proteasome-dependent destruction of the virus.[70,217] Interestingly, TRIM5α from Old World monkey confers potent resistance to HIV-1, but not SIV, while the human ortholog of TRIM5α is unable to specifically target HIV. The differential ability of the human and monkey TRIM5α to restrict HIV hinges on a single amino acid difference between the two proteins. HIV and SIV have likely evolved in their natural hosts to evade interaction with TRIM5α.

Inhibition of Viral Protein or Synthesis

Translation of viral genomes or virally encoded mRNAs is one of the major antiviral strategies used by various ISG effectors. The classic example is protein kinase R (PKR, gene *EIF2AK2*), a serine/threonine kinase that phosphorylates the α-subunit of eukaryotic translation initiation factor 2 an (eIF2α). This results in inhibition of translation and a decrease in total cellular and viral protein synthesis, effectively impairing viral production. In addition to its translational regulatory function, PKR has roles in signal transduction, transcriptional control, apoptosis, and autophagy, all of which can contribute to curbing viral replication and spread.[123,194]

The interferon-inducible tetratricopeptide (IFIT) proteins are also key ISG regulators of viral protein synthesis. Most mammals have five IFIT genes: *IFIT1*, *IFIT1B*, *IFIT2*, *IFIT3*, and *IFIT5*, many of which are among the most highly induced ISGs during an IFN response. In general, the IFIT proteins bind and sequester the 5′ ends of the viral RNA.[1,183] They outcompete eIF4F for binding to the RNA, effectively inhibiting translation. These proteins accommodate viral RNAs with unique structures that signal to the host cell that the RNAs are nonself. For example, human IFIT1 recognizes uncapped triphosphate RNA or capped RNAs lacking 2′*O*-methylation, which is the hallmark of most cellular RNAs.[50] Indeed, many viruses encode their own 2′*O*-methyltransferase as a mechanism to evade IFIT1-mediated restriction.

Inhibition of Viral Genome Nuclear Translocation

Many double-stranded DNA viruses, retroviruses, and certain RNA viruses like influenza A virus replicate their genomes in the nucleus. These genomes must translocate from the cytosol to the nucleus, and this process is the known target of ISG effectors MX1 and MX2. These proteins have a large N-terminal GTPase domain, a central interacting domain (CID), and a C-terminal leucine zipper (LZ) domain. Both the CID and the LZ domain are required to recognize target viral structures. MX1 inhibits nuclear translocation of influenza A virus genome, whereas MX2 analogously inhibits retroviruses and herpesvirus genomes from entering the nucleus.[79,84,87,106] It is interesting to note that most inbred strains of mice harbor a defective Mx1 gene.[83] Therefore, studies using inbred mice must be interpreted with the caveat that they are Mx1-deficient.

Inhibition of Late Stages

As with the early stages of the viral replication cycle, late stages can also be targeted by antiviral effectors. The classic example of a late-stage inhibitor is the GPI-anchored protein tetherin (gene *BST2*). Tetherin was first identified as a restriction factor that inhibits retroviral budding by physically tethering the outgoing virion to the viral membrane.[168] Tetherin has since been found to inhibit the egress of other viruses including influenza A virus, vesicular stomatitis virus, and hepatitis B virus.[37]

Multifunctional Antiviral Effectors

Not all ISG effectors fall neatly into the categories listed above. Some effectors have been reported to have antiviral effects at multiple steps in the viral replication cycle, or their mechanisms are indirect, or they are insufficiently clarified. Examples of seemingly multifunctional and or

“indirect” antiviral effectors are the zinc antiviral protein ZAP, viperin, OAS (2′-5′ oligoadenylate synthetase) proteins, and ISG15.

In humans, ZAP (gene *ZC3HAV1*) is expressed as two isoforms, one long (ZAP-L) and one short (ZAP-S). ZAP-L is longer due to the presence of a nonenzymatic PARP (poly ADP-ribose polymerase)-like domain and is considered the more antiviral of the two isoforms. ZAP proteins have broad activity against diverse viruses, including alphaviruses, filoviruses, and retroviruses. Multiple mechanisms of action have been reported for ZAP, including (1) targeting viral RNA to exosomes for degradation, (2) inhibiting translation of viral RNA, and (3) indirectly amplifying interferon induction pathways.[71,244] Notably, ZAP has specificity for viral RNAs enriched with CG dinucleotides, which are suppressed in vertebrate host genomes.[224]

Viperin (gene *RSAD2*) contains a radical *S*-adenosyl- l-methionine (SAM) domain and exhibits antiviral activity against a range of viruses including cytomegalovirus, influenza A virus, flaviviruses, and bunyaviruses.[42,191] One mechanism of action of viperin is to catalyze the formation of the small molecule 3′-deoxy-3′,4′-didehydro-CTP (ddhCTP), which functions as a chain terminator of the viral RNA-dependent RNA polymerase (RdRP).[76] Other studies report that viperin can inhibit viral replication by interfering with viral nonstructural proteins or interfere with egress by perturbing cellular membranes. Remarkably, viperin is not specific to eukaryotes. Ancient homologs of viperin have been identified in bacterial and archaeal genomes, and these molecules exert antiviral activity against phage.[23]

The oligoadenylate synthetases (encoded by *OAS1*, *OAS2*, *OAS3*) are three enzymes that are activated upon sensing viral RNA. The enzymes use ATP as a substrate and convert it into 2′-5′-linked oligoadenylates (2-5A). The 2-5A then activates latent RNAseL, which directly degrades viral genomes.[91,265] Notably, OAS1 exists predominantly as two major isoforms (p46 and p42) in the human population, due to genetic variation that results in differentially spliced transcripts. The p46 isoform is targeted to membranes via prenylation and inhibits viruses that co-opt host membranes to form their replication organelles (eg, flavivirus, coronavirus, etc.).[213,242] A fourth member of this family, OASL (gene *OASL*), does not have oligoadenylate synthetase activity, but rather modifies the activities of viral sensors by augmenting RIG-I or impairing cGAS pathways.[73,264]

ISG15 (gene *ISG15*) is a ubiquitin-like molecule that has many diverse functions related to the antiviral defense.[66] Like ubiquitin, ISG15 can be covalently attached to target proteins in a process called ISGylation, using E1 activating enzyme (UBE1L), E2 conjugating enzyme (UBCH8), and E3 ligases (HERC5, EFP, or TRIM25). The ubiquitin-specific protease USP18, also an ISG, can remove ISG15 modifications. ISGylation can occur on a variety of host and viral proteins, modifying the targets in a manner that generally disfavors some critical steps of the viral replication cycle. ISG15 can also be found in a free form that negatively regulates IFN induction and signaling, and a secreted form that functions as a signaling molecule to induce other cytokines such as IFN-γ.

Cell Types Involved in Innate Immune Responses to Virus Infections

In addition to the cell-intrinsic host defense mechanisms described above, mammals are equipped with many local and circulating immune cells that can provide further support to antiviral resistance mechanisms. Embedded in tissues are resident lymphocytes, dendritic cells, and macrophages that monitor the environment for invading pathogens. They provide local antiviral defense within minutes to hours of infection. In addition to the red blood cells and plasma, the peripheral blood contains white blood cells such as neutrophils, eosinophils, basophils, monocytes, dendritic cells, plasmacytoid dendritic cells, natural killer (NK) cells, and T and B lymphocytes. These cells can be called into the site of infection by chemokines produced by the local cells. T and B lymphocytes provide immune defense specific to the pathogen and are covered by Chapter 10. The circulating cells strengthen the innate immune response to viruses by inducing high levels of IFNs or killing virally infected cells.

Tissue-Resident Innate Immune Cells That Support Antiviral Defense

Within most peripheral tissues, there are lymphocytes and myeloid cells that are situated to monitor invading pathogens. This section discusses the relative roles of such tissue-resident leukocytes that confer antiviral immunity at the site of viral entry and replication.

Tissue-Resident Lymphocytes

These tissue-resident lymphocytes consist of both adaptive lymphocytes (conventional T cells) and innate or innate-like lymphocytes. They perform very similar functions of eliminating infected or damaged epithelial cells, but the initiating triggers differ. While conventional T cells detect viral antigens through their T-cell receptor (TCR) that recognize viral peptides presented on MHC molecules, innate and innatelike lymphocytes detect virus-infected cells through invariant TCR or TCR-independent manner through cytokine activation. Innate lymphocytes and innate-like lymphocytes are located either within or beneath the epithelial layer and detect signs of stress by the epithelial cells. These include cells with TCRs, such a γΔ T cells with invariant TCRs, mucosal-associated invariant T cells (MAIT cells), and NKT cells. Innate lymphocytes without TCRs include natural killer (NK) cells and innate lymphoid cells (ILC) (Fig. 9.10). MAIT cells express unique TCR and are restricted to the nonpolymorphic MHC class I–related 1 molecule (MR1), which recognizes vitamin B–based antigens. However, the response of MAIT cells to viruses does not rely on MR1 but the cytokines (especially IL-18, IL-12, IL-15, and type I IFNs) induced by myeloid cells in response to the virus.[77] Upon cytokine stimulation, MAIT cells, in turn, secrete IFN-γ and TNF and mediate cytotoxic effects, which may aid in the clearance of infected cells. ILCs consist of heterogeneous groups of innate lymphocytes that do not express antigen receptors. Group 1 ILCs consist of both mature NK cells and type 1 innate lymphoid cells (ILC1). They are further defined based on the production of the signature cytokine IFN-γ in response to the proinflammatory cytokines IL-12 and IL-18. Group I ILCs exert antiviral functions through the action of IFN-γ that induce hundreds of ISGs as discussed above. ILC1 is tissue resident and serves as a local source for IFN-γ early during infection (Fig. 9.10).

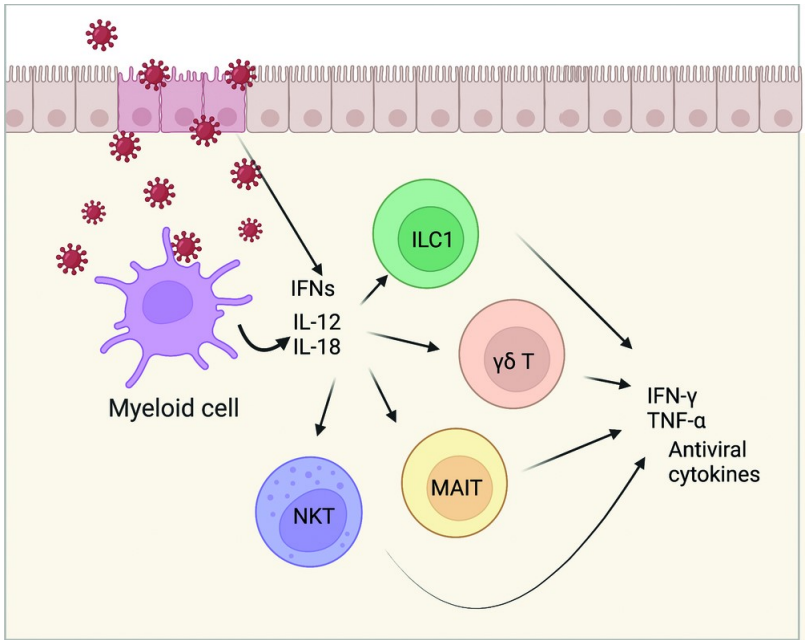

FIGURE 9.10 **Tissue-resident innate lymphocytes that contribute to antiviral defense**. Within peripheral tissues, several innate and innatelike lymphocytes, including ILC1, γΔT cells, MAIT, and NKT cells, are present. Upon stimulation by cytokines such as IL-12, IL-18, and type I and type III IFNs, these local innate lymphocytes secrete antiviral cytokines such as IFN-γ and TNF. These cytokines induce downstream antiviral genes to block the further spread of the virus before the adaptive immunity can be engaged.

Macrophages

Macrophages are tissue-resident myeloid cells that play central roles in host defense, inflammation, wound healing, tissue homeostasis, and tissue remodeling. Macrophages are professional phagocytes, clearing dying cells and pathogens. Macrophages can be found in most organs as specialized cell types, such as Kupffer cells (liver), microglia (neuronal tissue), and osteoclasts (bone). Macrophages are well known in their defense against bacterial and protozoan pathogens, as they are the chief phagocytes that engulf and degrade these types of pathogens upon activation with IFN-g.

Macrophages also play crucial roles in antiviral defense. Macrophages are situated at the forefront within mucosal barriers. In the lung, alveolar macrophages sample and degrade incoming pathogens. In addition, alveolar macrophages become highly infected with respiratory viruses such as Newcastle disease virus (NDV) and produce the highest levels of type I IFNs locally that prevent other cells from becoming infected.[122] Not only do macrophages provide the initial type I IFNs in infected tissue, but they also serve as a “viral sink” to prevent more vulnerable cell types from becoming infected. Subcutaneous injection of VSV results in a selective and productive infection of subcapsular sinus macrophages in the draining lymph nodes. These macrophages secrete high levels of type I IFNs that prevent infection of neurons. In mice depleted of such macrophages, local injection of VSV results in infection of innervating neurons in the lymph node leading to neuropathogenesis and death of mice.[93] Subcapsular macrophages also prevent systemic dissemination of lymph-borne pathogen by initiating an inflammatory immune response in the subcapsular region to activate and recruit innate and adaptive immune cells.[162]

Dendritic Cells

Dendritic cells are professional antigen-presenting cells capable of stimulating naïve antigen-specific lymphocytes in secondary lymphoid organs. Dendritic cells play an important role in immune responses at multiple levels. First, they are situated at various sites of pathogen entry and are among the first cells to recognize the incoming pathogens through a set of PRRs. Engagement of PRRs upon viral recognition induces the expression of genes required to both eliminate the pathogens (innate effectors) and to initiate adaptive immune responses.[157] Second, DCs are the only cell type capable of initiating adaptive immune responses by activating naïve T lymphocytes. Pathogen recognition through PRRs activates DCs to increase their expression of the chemokine receptor, CCR7, which enables them to migrate from the site of infection to the secondary lymphoid tissues, where naïve lymphocytes recirculate (Fig. 9.11). On transit, DCs undergo a maturation program that results in the upregulation of costimulatory molecules and translocation of their MHC class II to the cell surface.[233] Once in the lymph node, DCs can present antigens derived from the pathogens to naïve T cells and induce their activation (through costimulation) and differentiation (through secretion of appropriate cytokines)[15] (Fig. 9.11). A naïve T cell requires three kinds of signals for activation and differentiation. First, a T cell must recognize the viral peptide presented by the MHC molecules. CD4 T cells bind to peptides presented by MHC class II, while CD8 T cells bind to peptides presented by MHC class I. Second, T cells must receive signals from costimulatory molecules on APCs. PRR stimulation of APCs results in the expression of costimulatory molecules including CD80 and CD86, which bind to CD28 on naïve T cells and provide this second signal that allows optimal clonal expansion. The third signal dictates the differentiation of a naïve T cell into different effector types. CD4 T cells can differentiate into Th1, Th2, and Th17 cells depending on the cytokines provided by the APCs or other accessory cell types. Antiviral responses are mediated by Th1 cells, as they are uniquely capable of secreting IFN-γ.

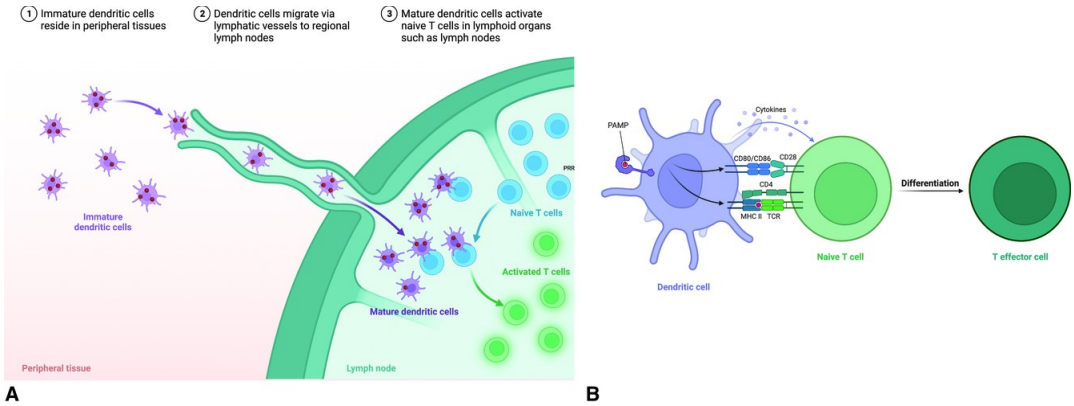

FIGURE 9.11 **Innate instruction of adaptive immunity**. **A**. Dendritic detect and phagocytose pathogens. DCs migrate to the lymph node, where they present viral antigenic peptides on MHC class I and MHC class II to activate naïve CD8 T and CD4 T cells whose T-cell receptors (TCR) are specific to the peptide presented by MHC molecules, respectively. **B**. PRR engagement (shown here for a TLR) by viral PAMPs induce expression of costimulatory molecules CD80 and CD86, as well as cytokines that stimulate naïve T cells, providing second and third signals needed for robust T-cell activation and differentiation. T cells receiving all three signals can proliferate and differentiate to become effector T cells. Depending on the cytokines secreted by DCs, CD4 T cells can become various effector T-cell types.

DCs can be broadly divided into two types, those that reside in the peripheral tissues (mucosa, skin, internal organs) and those that reside in the blood and lymphoid tissues.[98] Different DC subsets orchestrate innate and adaptive immune responses to viruses, depending on the type of PRR engaged by a given virus infection (see Chapter 10, The Adaptive Immune Response to Viruses).

Recruited Innate Immune Cells for Antiviral Defense

In addition to tissue-resident leukocytes, circulating immune cells are called into the site of infection to prevent further spread of the virus.

Natural Killer Cells

Unlike ILC1, NK cells are circulating and are recruited to the sites of infection to mediate antiviral defense. NK cells are activated by cytokines, as well as cells that express NK-activating receptor ligands and those that express lower levels of MHC class I. NK cells express two classes of receptors, known as activating and inhibitory receptors. These receptors induce or inhibit the target cell lysis and IFN-γ production upon recognition of their cognate ligands on target cells (Fig. 9.12). The ligands for activating NK receptors are induced in virally infected cells. The ligands for NK inhibitory receptors are MHC class I molecules and other molecules constitutively expressed on most cells and downregulated upon viral infection. The ligands for inhibitory receptors can be downregulated either as a result of sensing the viral infection in the cell or, in the case of MHC I, as a result of viral downregulation of MHC I. Many DNA viruses encode molecules dedicated for evasion of MHC I processing and presentation of viral antigens, to avoid recognition by CD8 T cells.[85] Through their ability to detect missing self (reduction in MHC class I molecules on the cell surface), NK cells fulfill the gap in immunosurveillance by CD8 T cells. Thus, NK cell recognition of infected cells relies on both the sensing of “induced self” (by activating receptors) and the “missing self” (by inhibitory receptors)[186] (Fig. 9.12). The net effect of these changes is detected by NK cells. Only when positive signals dominate, NK cells induce apoptotic death of target cells by unleashing their cytotoxic granules.

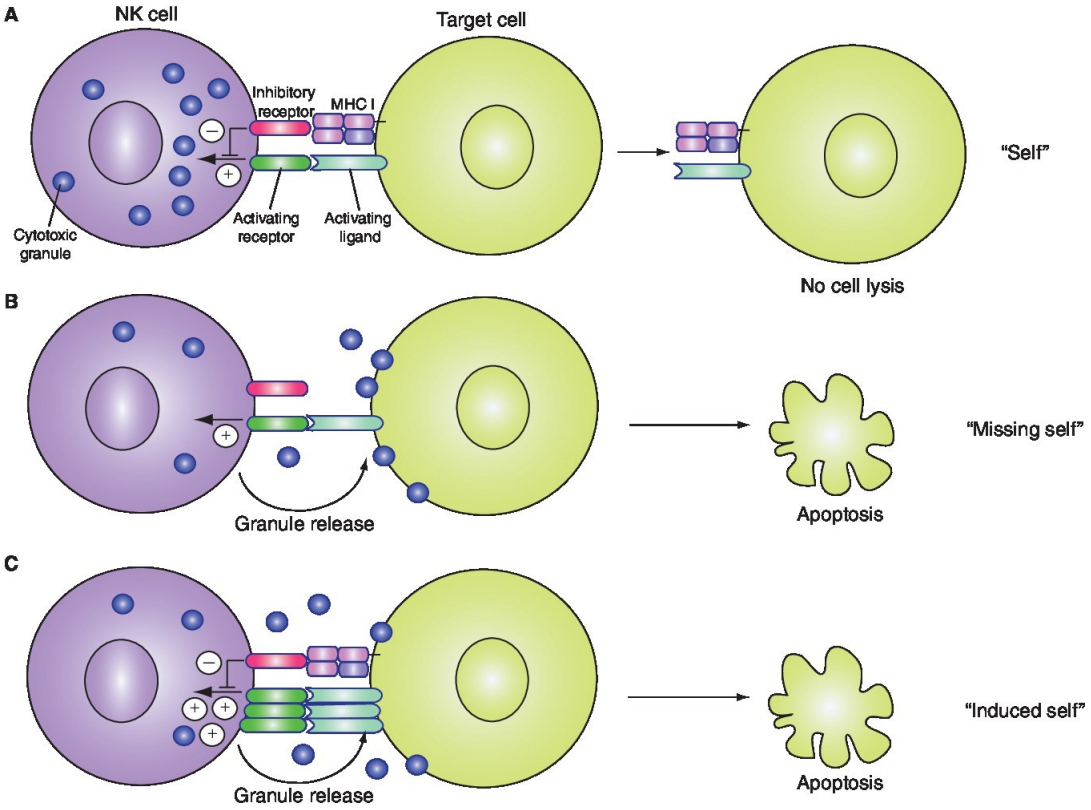

FIGURE 9.12 **Natural killer cell activation and inhibition**. NK cell recognition of infected cells relies on both the sensing of "induced self" (by activating receptors) and the "missing self" (by inhibitory receptors). NK cells contain cytosolic lytic granules that mediate apoptosis in target cells. **A**. When an NK cell recognizes a healthy cell, the NK cell lysis of the target cell is prevented by engagement of the inhibitory receptor by normal level of MHC class I on the target cell. **B**. When an NK cell interacts with virally infected cells that have reduced MHC class I on the cell surface ("missing self"), NK cells induce apoptosis in the target cell. **C**. When an NK cell recognizes stressed virally infected cell expressing high levels of stimulating ligands ("induced self"), negative signals from the inhibitory receptor is overridden by the activating signal, leading to lysis of target cells.

The ligands for NK activating receptors are generally absent or expressed at a low level on normal uninfected cells. Their expression is upregulated in response to viral infection and cellular stress. The best characterized activating ligands are recognized by NKG2D.[126] Human ligands for NKG2D include MHC class-I-chain–related protein A (MICA) and MICB, which are nonclassical MHC I molecules. In mice, NKG2D binds to several ligands including retinoic acid early transcript 1 (Rae1), a glycosylphosphatidylinositol (GPI) anchored protein, histocompatibility 60 (H60), and mouse UL16-binding proteinlike transcript 1 (Mult1). While Rae1, H60, and Mult1 only share 20% amino acid sequence homology, they share a structurally related domain, which is distantly related to MHC I a1 and a2 domains. Inducible expression of these ligands can overcome inhibitory signals provided by recognition of normally expressed MHC class I molecules, resulting in target cell lysis (Fig. 9.12C).

NK inhibitory receptors sense the level of MHC class I molecules on target cells. The CD94:NKG2A heterodimers recognize nonpolymorphic MHC class I molecules HLA-E in humans and Qa-1 in mice, which present leader peptides from classical MHC class I proteins. Thus, when the MHC I levels decrease as a result of viral manipulation, it is reflected in the decreased level of HLA-E/Qa-1 expression, resulting in disengagement of the CD94:NKG2A inhibitory receptor. Not surprisingly, several viruses mimic the activity of HLA-E. UL40 of HCMV confers protection in infected cells by encoding the same leader sequence that can be presented on HLA-E,[231] while HIV-1 selectively avoids downregulation of HLA-E and HLA-C to prevent NK-mediated lysis.[46] Mouse Ly49 family of inhibitory receptors recognizes the polymorphic MHC class I molecules. There are 18 Ly49 proteins in mice that have specificity to different MHC class I haplotypes. This mechanism of NK inhibition has been co-opted by the MCMV protein m157, which stimulates Ly49I to inhibit NK activation.[11]

Plasmacytoid Dendritic Cells

Plasmacytoid dendritic cells are considered professional viral sensors for several reasons.[141] First, pDCs are equipped with special cellular machinery to endocytose many types of viruses and transport them to the endolysosomal compartment, whereupon uncoating of viral envelope and capsid, viral genomic DNA, or RNA can be detected through TLR9 or TLR7, respectively. Second, pDCs constitutively express molecules involved in signaling to induce type I IFN genes. For instance, these cells express constitutively high levels of IRF7[112] and utilize various signaling proteins downstream of TLRs to induce IFN transcription. Third, pDCs form specialized lysosome-related organelles from which TLR9 and TLR7 can engage IRF7 activation to trigger the transcription of type I IFN genes.

Consequently, pDCs were originally known as IFN-producing cells (IPC), characterized by their potent ability to secrete type I IFNs in response to viruses.[13,28,74,99] Whereas DCs as a class are thought to be the professional antigen-presenting cells specializing in antigen uptake, processing, and presentation to T cells, the predominant role of the pDCs appears to be the secretion of type I IFNs. In particular, pDCs can be induced to secrete large amounts of IFN-α when stimulated with many DNA and RNA viruses. However, this situation may not be universal, as inflammatory monocytes and alveolar macrophages are the predominant IFN producers after vaccinia virus or NDV infection, respectively.[16122]

The pDCs express both L-selectin and CCR7 and can enter from the blood vessel into secondary lymphoid tissues.[35] At a steady state, these cells are not found in peripheral tissues. However, they can be induced to migrate into peripheral tissues and secrete antiviral IFNs *in situ*.[149] In mice that are depleted of pDCs, virus infections with MCMV and VSV resulted in reduced early IFN-I production and increased viral burden leading to impaired survival.[219] Given the location of pDCs, these cells likely serve as the initial source for type I IFNs in response to systemic viral infections, and as a secondary source of type I IFNs in response to localized viral infections restricted to the peripheral tissues.

Monocytes

Circulating monocytes also play an important role during viral infection. Monocytes patrol the bloodstream and peripheral tissue for infection. Once inside the tissue, monocytes can give rise to macrophages and dendritic cells. Circulating monocytes can be broadly categorized into two types, inflammatory monocytes and patrolling monocytes.[14] Inflammatory monocytes ($Ly6C^+$ $CCR2^+$ in mice and CD14 hi CD16lo in humans) are selectively recruited to inflamed tissues and lymph nodes, producing high levels of TNF-α and IL-1 during infection. Mouse inflammatory monocytes express TLR2 and recognize dsDNA viruses such as vaccinia virus and MCMV and secrete large amounts of type I IFNs.[16] Interestingly, monocyte recognition of viruses by TLR2 requires endocytosis while recognition of bacterial TLR2 ligands does not. While the exact viral ligands for TLR2-dependent detection by monocytes is unclear, TLR2 is known to be triggered by a variety of viruses including human cytomegalovirus,[47] mouse cytomegalovirus (MCMV),[220] herpes simplex virus types 1 and 2,[125,197] hepatitis C virus,[36] lymphocytic choriomeningitis virus,[262] measles virus,[24] and vaccinia virus.[263]

The other subset of monocytes ($Ly6C^-$ in mice and $CD14^{dim}$ CD16hi in humans) patrols the blood vasculature, can differentiate into macrophages after extravasation into tissues, and may promote tissue repair.[14] The human $CD14^{dim}$ monocytes do not express cell surface TLRs but express endosomal TLR7 and TLR8 and after recognition of viral nucleic acids produce TNF, IL-1β, and CCL3, but not type I IFNs.[48] Thus, monocytes as a class of cells promote antiviral resistance through the secretion of IFNs and other cytokines that induce antiviral genes in neighboring cells, as well as promote the recruitment of adaptive immune cells for viral clearance.

Viral Evasion of Innate Immunity

The innate immune response is a formidable evolutionary pressure on viruses. Accordingly, virtually all viruses have evolved mechanisms to counteract the induction, signaling, or antiviral effector functions induced by antiviral IFNs.[239] As a vast literature covers this topic, only key concepts with select examples will be presented here.

Viruses can interfere with initial host sensing at the level of PRRs. For example, paramyxovirus V protein prevents MDA5 dimerization,[10] whereas influenza A and B viruses express an NS1 protein preventing the activation of RIG-I.[69] Notably, NS1 also has other evasion mechanisms including inhibition of host mRNA processing and export, blockade of IFNAR signaling, and antagonism of innate effectors PKR and OAS.[239] PRR adaptors can also be targeted, with hepatitis C virus NS3 protease-mediated cleavage of MAVS serving as a prime example.[132,161]

A common viral strategy to interfere with IFN gene induction is represented by viral inhibitors of IRF3 and IRF7. Examples include KSHV vIRF3 that blocks the host IRF7 activity[105] and rotavirus NSP1 that blocks IRF3 and IRF7.[18,206] Even if the infected cells can produce type I IFNs, many viruses encode factors that inhibit signaling through the IFNAR. SARS-CoV and SARS-CoV-2 ORF6 protein prevent nuclear translocation of STAT1 by sequestering nuclear import factors on the ER/Golgi membrane.[68] Lastly, ISG effectors themselves can also be targets of viral evasion. For example, certain viruses can antagonize tetherin-mediated inhibition of viral egresses, such as HIV-1 with its Vpu protein.[168]

Differences Between Evasion Strategy Mechanisms of DNA vs RNA Viruses

Even though all viruses encode factors that interfere with host antiviral defense mechanisms, different classes of viruses appear to target different stages in antiviral defense. Nearly all viruses with (−)ssRNA genomes interfere with the innate sensors themselves. By contrast, (+)ssRNA and dsDNA viruses target the downstream IRFs as the means to interfere with IFN induction.[239] Another intriguing feature shared among the dsDNA viruses, particularly poxviruses, is that they encode soluble decoy molecules that antagonize antiviral signaling to prevent neighboring cells from becoming resistant to viral infection. Large dsDNA viruses can also accommodate multiple genes to specifically inhibit pathways that are useful for their replicative niche. By expressing soluble IFN-binding proteins that compete with the cellular IFN receptor for its ligand, dsDNA viruses allow for infection and spread to cells that would otherwise be prevented from infection. Another feature associated with dsDNA viruses is their ability to establish latency. To this end, almost all herpesviruses encode molecules that specifically block PKR and its downstream target to block the cell-mediated arrest of translation. This strategy is particularly important to ensure the survival of the latently infected cells. Other dsDNA viruses have narrow targets such as a single NK receptor ligand. MCMV encodes three proteins, m152, m145, and m155, that reduce surface expression of NKG2D ligands, RAE-1,[142] MULT-1,[120] and H60,[143] respectively, in infected cells. Chronicity of virus infection may result from the ability of dsDNA viruses to manipulate the host immune response by interfering with specific effector mechanisms of various host cell types.

Conclusion

In the past 10 years, there has been tremendous progress in the characterization of the innate immune recognition of viral pathogens. The role of type I IFNs, NK cells, TLRs, cGAS, and RLRs in antiviral defense is now well appreciated and understood in considerable detail. Many IFN-induced antiviral gene products still have to be functionally characterized. These proteins likely interfere with multiple steps in viral infection cycles. This functional redundancy makes the analysis of their contribution to antiviral defense particularly challenging. Other outstanding questions include the impact of host-intrinsic factors, such as aging and biological sex, as well as the impact of the host-extrinsic factors, such as the environment and diet, on antiviral innate immune defense. Finally, how a viral infection could lead to chronic disease conditions, such as autoimmunity and neurodegenerative disease, remains a fruitful area of research. Ultimately, the greatest challenge lies with the application of the accumulating knowledge to the management of emerging pandemics and the ultimate eradication of major viral infections that continue to threaten our well-being.

REFERENCES

1. Abbas YM, Pichlmair A, Gorna MW, Superti-Furga G, Nagar B. Structural basis for viral 5′-PPP-RNA recognition by human IFIT proteins. Nature. 2013;494:60-64.

2. Ablasser A, Bauernfeind F, Hartmann G, Latz E, Fitzgerald KA, Hornung V. RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. Nat Immunol. 2009;10:1065-1072.

3. Ablasser A, Goldeck M, Cavlar T, et al. cGAS produces a 2′-5′-linked cyclic dinucleotide second messenger that activates STING. Nature. 2013;498:380-384.

4. Ahmad S, Mu X, Yang F, et al. Breaching self-tolerance to Alu duplex RNA underlies MDA5-mediated inflammation. Cell. 2018;172:797-810.

5. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2012;4:499-511.

6. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001;413:732-738.

7. Allen IC, Scull MA, Moore CB, et al. The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. Immunity. 2009;30: 556-565.

8. Andreeva L, David L, Rawson S, et al. NLRP3 cages revealed by full-length mouse NLRP3 structure control pathway activation. Cell. 2021;184:6299-6312 e6222.

9. Andreeva L, Hiller B, Kostrewa D, et al. cGAS senses long and HMGB/TFAM-bound U-turn DNA by forming protein-DNA ladders. Nature. 2017;549:394-398.

10. Andrejeva J, Childs KS, Young DF, et al. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. Proc Natl Acad Sci U S A. 2004;101:17264-17269.

11. Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science. 2002;296:1323-1326.

12. Areschoug T, Gordon S. Pattern recognition receptors and their role in innate immunity: focus on microbial protein ligands. Contrib Microbiol. 2008;15:45-60.

13. Asselin-Paturel C, Boonstra A, Dalod M, et al. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. Nat Immunol. 2001;2:1144-1150.

14. Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu Rev Immunol. 2009;27:669-692.

15. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392:245-252.

16. Barbalat R, Lau L, Locksley RM, Barton GM. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. Nat Immunol. 2009;10:1200-1207.

17. Baribaud F, Doms RW, Pohlmann S. The role of DC-SIGN and DC-SIGNR in HIV and Ebola virus infection: can potential therapeutics block virus transmission and dissemination? Expert Opin Ther Targets. 2002;6:423-431.

18. Barro M, Patton JT. Rotavirus NSP1 inhibits expression of type I interferon by antagonizing the function of interferon regulatory factors IRF3, IRF5, and IRF7. J Virol. 2007;81:4473-4481.

19. Barton GM, Kagan JC, Medzhitov R. Intracellular localization of toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nat Immunol. 2006;7:49-56.

20. Baum A, Sachidanandam R, Garcia-Sastre A. Preference of RIG-I for short viral RNA molecules in infected cells revealed by next-generation sequencing. Proc Natl Acad Sci U S A. 2010;108:3092.

21. Ben-Sasson SZ, Hu-Li J, Quiel J, et al. IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. Proc Natl Acad Sci U S A. 2009;106:7119-7124.

22. Berke IC, Yu X, Modis Y, Egelman EH. MDA5 assembles into a polar helical filament on dsRNA. Proc Natl Acad Sci U S A. 2012;109:18437-18441.

23. Bernheim A, Millman A, Ofir G, et al. Prokaryotic viperins produce diverse antiviral molecules. Nature. 2021;589:120-124.

24. Bieback K, Lien E, Klagge IM, et al. Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling. J Virol. 2002;76:8729-8736.

25. Bonham KS, Orzalli MH, Hayashi K, et al. A promiscuous lipid-binding protein diversifies the subcellular sites of toll-like receptor signal transduction. Cell. 2014;156:705-716.

26. Boyer JA, Spangler CJ, Strauss JD, et al. Structural basis of nucleosome-dependent cGAS inhibition. Science. 2020;370:450-454.

27. Brass AL, Huang IC, Benita Y, et al. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. Cell. 2009;139:1243-1254.

28. Brawand P, Fitzpatrick DR, Greenfield BW, Brasel K, Maliszewski CR, De Smedt T. Murine plasmacytoid pre-dendritic cells generated from flt3 ligand-supplemented bone marrow cultures are immature APCs. J Immunol. 2002;169:6711-6719.

29. Brinkmann MM, Spooner E, Hoebe K, Beutler B, Ploegh HL, Kim YM. The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling. J Cell Biol. 2007;177:265-275.

30. Bruns AM, Leser GP, Lamb RA, Horvath CM. The innate immune sensor LGP2 activates antiviral signaling by regulating MDA5-RNA interaction and filament assembly. Mol Cell. 2014;55:771-781.

31. Burckstummer T, Baumann C, Bluml S, et al. An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. Nat Immunol. 2009;10(3):266-272.

32. Cadena C, Ahmad S, Xavier A, et al. Ubiquitin-dependent and -independent roles of E3 ligase RIPLET in innate immunity. Cell. 2019;177:1187-1200 e1116.

33. Cao D, Han X, Fan X, Xu RM, Zhang X. Structural basis for nucleosome-mediated inhibition of cGAS activity. Cell Res. 2020;30:1088-1097.

34. Casrouge A, Zhang SY, Eidenschenk C, et al. Herpes simplex virus encephalitis in human UNC-93B deficiency. Science. 2006;314:308-312.

35. Cella M, Jarrossay D, Facchetti F, et al. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nat Med. 1999;5:919-923.

36. Chang S, Dolganiuc A, Szabo G. Toll-like receptors 1 and 6 are involved in TLR2-mediated macrophage activation by hepatitis C virus core and NS3 proteins. J Leukoc Biol. 2007;82:479-487.

37. Chemudupati M, Kenney AD, Bonifati S, et al. From APOBEC to ZAP: diverse mechanisms used by cellular restriction factors to inhibit virus infections. Biochim Biophys Acta Mol Cell Res. 2019;1866:382-394.

38. Chen J, Chen ZJ. PtdIns4P on dispersed trans-Golgi network mediates NLRP3 inflammasome activation. Nature. 2018;564:71-76.

39. Chen ST, Lin YL, Huang MT, et al. CLEC5A is critical for dengue-virus-induced lethal disease. Nature. 2008;453:672-676.

40. Chiappinelli KB, Strissel PL, Desrichard A, et al. Inhibiting DNA methylation causes an interferon response in cancer via dsRNA including endogenous retroviruses. Cell. 2015;162:974-986.

41. Childs KS, Randall RE, Goodbourn S. LGP2 plays a critical role in sensitizing mda-5 to activation by double-stranded RNA. PLoS One. 2013;8:e64202.

42. Chin KC, Cresswell P. Viperin (cig5), an IFN-inducible antiviral protein directly induced by human cytomegalovirus. Proc Natl Acad Sci U S A. 2001;98:15125-15130.

43. Chiu YH, Macmillan JB, Chen ZJ. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. Cell. 2009;138:576-591.

44. Cho YS, Challa S, Moquin D, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell. 2009;137:1112-1123.

45. Clabbers MTB, Holmes S, Muusse TW, et al. MyD88 TIR domain higher-order assembly interactions revealed by microcrystal electron diffraction and serial femtosecond crystallography. Nat Commun. 2021;12:2578.

46. Cohen GB, Gandhi RT, Davis DM, et al. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity. 1999;10:661-671.

47. Compton T, Kurt-Jones EA, Boehme KW, et al. Human cytomegalovirus activates inflammatory cytokine responses via CD14 and toll-like receptor 2. J Virol. 2003;77:4588-4596.

48. Cros J, Cagnard N, Woollard K, et al. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. Immunity. 2010;33:375-386.

49. Crow YJ, Hayward BE, Parmar R, et al. Mutations in the gene encoding the 3′-5′ DNA exonuclease TREX1 cause Aicardi-Goutieres syndrome at the AGS1 locus. Nat Genet. 2006;38:917-920.

50. Daffis S, Szretter KJ, Schriewer J, et al. 2′-O methylation of the viral mRNA cap evades host restriction by IFIT family members. Nature. 2010;468:452-456.

51. de Witte L, Nabatov A, Pion M, et al. Langerin is a natural barrier to HIV-1 transmission by langerhans cells. Nat Med. 2007;13:367-371.

52. Demaria O, Pagni PP, Traub S, et al. TLR8 deficiency leads to autoimmunity in mice. J Clin Invest. 2010;120:3651-3662.

53. den Dunnen J, Gringhuis SI, Geijtenbeek TB. Innate signaling by the C-type lectin DC-SIGN dictates immune responses. Cancer Immunol Immunother. 2009;58:1149-1157.

54. Denton A, Doherty P, Turner S, La Gruta N. IL-18, but not IL-12, is required for optimal cytokine production by influenza virus-specific CD8+ T cells. Eur J Immunol. 2007;37:368-375.

55. Der SD, Zhou A, Williams BR, Silverman RH. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci U S A. 1998;95:15623-15628.

56. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science. 2004;303:1529-1531.

57. Dinarello CA. Biologic basis for interleukin-1 in disease. Blood. 1996;87:2095-2147.

58. Diner EJ, Burdette DL, Wilson SC, et al. The innate immune DNA sensor cGAS produces a noncanonical cyclic dinucleotide that activates human STING. Cell Rep. 2013;3:1355-1361.

59. Dixit E, Boulant S, Zhang Y, et al. Peroxisomes are signaling platforms for antiviral innate immunity. Cell. 2010;141:668-681.

60. Ergun SL, Fernandez D, Weiss TM, Li L. STING polymer structure reveals mechanisms for activation, hyperactivation, and inhibition. Cell. 2019;178:290-301.e210.

61. Ewald SE, Lee BL, Lau L, et al. The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor. Nature. 2008;456:658-662.

62. Fang R, Jiang Q, Zhou X, et al. MAVS activates TBK1 and IKKepsilon through TRAFs in NEMO dependent and independent manner. PLoS Pathog. 2017;13:e1006720.

63. Feng X, Tubbs A, Zhang C, et al. ATR inhibition potentiates ionizing radiation-induced interferon response via cytosolic nucleic acid-sensing pathways. EMBO J. 2020;39:e104036.

64. Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature. 2009;458(7237): 509-513.

65. Ferreira CR, Crow YJ, Gahl WA, et al. DDX58 and classic Singleton-Merten syndrome. J Clin Immunol. 2019;39:75-80.

66. Freitas BT, Scholte FEM, Bergeron E, Pegan SD. How ISG15 combats viral infection. Virus Res. 2020;286:198036.

67. French AR, Holroyd EB, Yang L, Kim S, Yokoyama WM. IL-18 acts synergistically with IL-15 in stimulating natural killer cell proliferation. Cytokine. 2006;35:229-234.

68. Frieman M, Yount B, Heise M, Kopecky-Bromberg SA, Palese P, Baric RS. Severe acute respiratory syndrome coronavirus ORF6 antagonizes STAT1 function by sequestering nuclear import factors on the rough endoplasmic reticulum/Golgi membrane. J Virol. 2007;81:9812-9824.

69. Gack MU, Albrecht RA, Urano T, et al. Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. Cell Host Microbe. 2009;5:439-449.

70. Ganser-Pornillos BK, Pornillos O. Restriction of HIV-1 and other retroviruses by TRIM5. Nat Rev Microbiol. 2019;17:546-556.

71. Gao G, Guo X, Goff SP. Inhibition of retroviral RNA production by ZAP, a CCCH-type zinc finger protein. Science. 2002;297:1703-1706.

72. Gao P, Ascano M, Wu Y, et al. Cyclic [G(2′,5′)pA(3′,5′)p] is the metazoan second messenger produced by DNA-activated cyclic GMP-AMP synthase. Cell. 2013;153:1094-1107.

73. Ghosh A, Shao L, Sampath P, et al. Oligoadenylate-synthetase-family protein OASL inhibits activity of the DNA sensor cGAS during DNA virus infection to limit interferon production. Immunity. 2019;50:51-63.e55.

74. Gilliet M, Boonstra A, Paturel C, et al. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. J Exp Med. 2002;195:953-958.

75. Gitlin L. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. Proc Natl Acad Sci U S A. 2006;103:8459.

76. Gizzi AS, Grove TL, Arnold JJ, et al. A naturally occurring antiviral ribonucleotide encoded by the human genome. Nature. 2018;558:610-614.

77. Godfrey DI, Koay H-F, McCluskey J, Gherardin NA. The biology and functional importance of MAIT cells. Nat Immunol. 2019;20:1110-1128.

78. Goubau D, Schlee M, Deddouche S, et al. Antiviral immunity via RIG-I-mediated recognition of RNA bearing 5′-diphosphates. Nature. 2014;514;372-375.

79. Goujon C, Moncorge O, Bauby H, et al. Human MX2 is an interferon-induced post-entry inhibitor of HIV-1 infection. Nature. 2013;502:559-562.

80. Greulich W, Wagner M, Gaidt MM, et al. TLR8 is a sensor of RNase T2 degradation products. Cell. 2019;179:1264-1275.e1213.

81. Gui X, Yang H, Li T, et al. Autophagy induction via STING trafficking is a primordial function of the cGAS pathway. Nature. 2019;567:262-266.

82. Haas T, Metzger J, Schmitz F, et al. The DNA sugar backbone 2′ deoxyribose determines toll-like receptor 9 activation. Immunity. 2008;28:315-323.

83. Haller O, Staeheli P, Kochs G. Interferon-induced Mx proteins in antiviral host defense. Biochimie. 2007;89:812-818.

84. Haller O, Staeheli P, Schwemmle M, Kochs G. Mx GTPases: dynamin-like antiviral machines of innate immunity. Trends Microbiol. 2015;23:154-163.

85. Hansen TH, Bouvier M. MHC class I antigen presentation: learning from viral evasion strategies. Nat Rev Immunol. 2009;9:503-513.

86. Hemmi H, Takeuchi O, Kawai T, et al. A Toll-like receptor recognizes bacterial DNA. Nature. 2000;408:740-745.

87. Horisberger MA, Staeheli P, Haller O. Interferon induces a unique protein in mouse cells bearing a gene for resistance to influenza virus. Proc Natl Acad Sci U S A. 1983;80:1910-1914.

88. Hornung V, Ablasser A, Charrel-Dennis M, et al. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature. 2009;458(7237):514-518.

89. Hoshino T, Kawase Y, Okamoto M, et al. Cutting edge: IL-18-transgenic mice: in vivo evidence of a broad role for IL-18 in modulating immune function. J Immunol. 2001;166:7014-7018.

90. Hou F, Sun L, Zheng H, Skaug B, XJiang QX, Chen ZJ. MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. Cell. 2011;146:1-14.

91. Hur S. Double-stranded RNA sensors and modulators in innate immunity. Annu Rev Immunol. 2019;37:349-375.

92. Hyodo Y, Matsui K, Hayashi N, et al. IL-18 up-regulates perforin-mediated NK activity without increasing perforin messenger RNA expression by binding to constitutively expressed IL-18 receptor. J Immunol. 1999;162:1662-1668.

93. Iannacone M, Moseman EA, Tonti E, et al. Subcapsular sinus macrophages prevent CNS invasion on peripheral infection with a neurotropic virus. Nature. 2010;465:1079.

94. Ichinohe T, Lee HK, Ogura Y, Flavell R, Iwasaki A. Inflammasome recognition of influenza virus is essential for adaptive immune responses. J Exp Med. 2009;206(1):79-87.

95. Ichinohe T, Pang IK, Iwasaki A. Influenza virus activates inflammasomes via its intracellular M2 ion channel. Nat Immunol. 2010;11:404-410.

96. Isaacs A, Lindenmann J. Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci. 1957;147:258-267.

97. Ishikawa H, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature. 2008;455:674-678.

98. Itano AA, Jenkins MK. Antigen presentation to naive CD4 T cells in the lymph node. Nat Immunol. 2003;4:733-739.

99. Ito T, Amakawa R, Kaisho T, et al. Interferon-alpha and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets. J Exp Med. 2002;195:1507-1512.

100. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol. 2004;5:987-995.

101. Jang M-A, Kim EK, Now H, et al. Mutations in DDX58, which encodes RIG-I, cause atypical singleton-merten syndrome. Am J Hum Genet. 2015;96:266-274.

102. Jeremiah N, Neven B, Gentili M, et al. Inherited STING-activating mutation underlies a familial inflammatory syndrome with lupus-like manifestations. J Clin Invest. 2014;124:5516-5520.

103. Jiang X, Kinch LN, Brautigam CA, et al. Ubiquitin-induced oligomerization of the RNA sensors RIG-I and MDA5 activates antiviral innate immune response. Immunity. 2012;36:959-973.

104. Jin T, Perry A, Jiang J, et al. Structures of the HIN domain:DNA complexes reveal ligand binding and activation mechanisms of the AIM2 inflammasome and IFI16 receptor. Immunity. 2012;36:561-571.

105. Joo CH, Shin YC, Gack M, Wu L, Levy D, Jung JU. Inhibition of interferon regulatory factor 7 (IRF7)-mediated interferon signal transduction by the Kaposi's sarcoma-associated herpesvirus viral IRF homolog vIRF3. J Virol. 2007;81:8282-8292.

106. Kane M, Yadav SS, Bitzegeio J, et al. MX2 is an interferon-induced inhibitor of HIV-1 infection. Nature. 2013;502:563-566.

107. Kanneganti TD, Body-Malapel M, Amer A, et al. Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. J Biol Chem. 2006;281:36560-36568.

108. Kanno A, Yamamoto C, Onji M, et al. Essential role for Toll-like receptor 7 (TLR7)-unique cysteines in an intramolecular disulfide bond, proteolytic cleavage and RNA sensing. Int Immunol. 2013;25:413-422.

109. Kato H, Takeuchi O, Mikamo-Satoh E, et al. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J Exp Med. 2008;205:1601-1610.

110. Kato H, Takeuchi O, Sato S, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature. 2006;441:101

111. Kato K, Ahmad S, Zhu Z, et al. Structural analysis of RIG-I-like receptors reveals ancient rules of engagement between diverse RNA helicases and TRIM ubiquitin ligases. Mol Cell. 2021;81:599-613.e598.

112. Kerkmann M, Rothenfusser S, Hornung V, et al. Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells. J Immunol. 2003;170:4465-4474.

113. Kerrigan AM, Brown GD. Syk-coupled C-type lectin receptors that mediate cellular activation via single tyrosine based activation motifs. Immunol Rev. 2010;234:335-352.

114. Kerur N, Veettil MV, Sharma-Walia N, et al. IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi Sarcoma-associated herpesvirus infection. Cell Host Microbe. 2011;9:363-375.

115. Kesavardhana S, Kuriakose T, Guy CS, et al. ZBP1/DAI ubiquitination and sensing of influenza vRNPs activate programmed cell death. J Exp Med. 2017;214:2217-2229.

116. Kim SY, Solomon DH. Tumor necrosis factor blockade and the risk of viral infection. Nat Rev Rheumatol. 2010;6(3):165-174. doi:10.1038/nrrheum.2009.279

117. Kishimoto T. Interleukin-6: from basic science to medicine—40 years in immunology. Annu Rev Immunol. 2005;23:1-21.

118. Kohlway A, Luo D, Rawling DC, Ding SC, Pyle AM. Defining the functional determinants for RNA surveillance by RIG-I. EMBO Rep. 2013;14:772-779.

119. Kotenko SV, Rivera A, Parker D, Durbin JE. Type III IFNs: beyond antiviral protection. Semin Immunol. 2019;43:101303.

120. Krmpotic A, Hasan M, Loewendorf A, et al. NK cell activation through the NKG2D ligand MULT-1 is selectively prevented by the glycoprotein encoded by mouse cytomegalovirus gene m145. J Exp Med. 2005;201:211-220.

121. Kruger A, Oldenburg M, Chebrolu C, et al. Human TLR8 senses UR/URR motifs in bacterial and mitochondrial RNA. EMBO Rep. 2015;16:1656-1663.

122. Kumagai Y, Takeuchi O, Kato H, et al. Alveolar macrophages are the primary interferon-alpha producer in pulmonary infection with RNA viruses. Immunity. 2007;27:240-252.

123. Kumar A, Haque J, Lacoste J, Hiscott J, Williams BR. Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B. Proc Natl Acad Sci U S A. 1994;91:6288-6292.

124. Kuriakose T, Man SM, Malireddi RK, et al. ZBP1/DAI is an innate sensor of influenza virus triggering the NLRP3 inflammasome and programmed cell death pathways. Sci Immunol. 2016;1(2):aag2045.

125. Kurt-Jones EA, Chan M, Zhou S, et al. Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. Proc Natl Acad Sci U S A. 2004;101:1315-1320.

126. Lanier LL. NK cell recognition. Annu Rev Immunol. 2005;23:225.

127. Lazear HM, Schoggins JW, Diamond MS. Shared and distinct functions of type I and type III interferons. Immunity. 2019;50:907-923.

128. Lee HK, Lund JM, Ramanathan B, Mizushima N, Iwasaki A. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. Science. 2007;315:1398-1401.

129. Lenoir JJ, Parisien JP, Horvath CM. Immune regulator LGP2 targets Ubc13/UBE2N to mediate widespread interference with K63 polyubiquitination and NF-kappaB activation. Cell Rep. 2021;37:110175.

130. Leonard JN, Ghirlando R, Askins J, et al. The TLR3 signaling complex forms by cooperative receptor dimerization. Proc Natl Acad Sci U S A. 2008;105:258-263.

131. Li T, Diner BA, Chen J, Cristea IM. Acetylation modulates cellular distribution and DNA sensing ability of interferon-inducible protein IFI16. Proc Natl Acad Sci U S A. 2012;109:10558-10563.

132. Li X, Shu C, Yi G, et al. Cyclic GMP-AMP synthase is activated by double-stranded DNA-induced oligomerization. Immunity. 2013;39:1019-1031.

133. Lin SC, Lo YC, Wu H. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. Nature. 2010;465:885-890.

134. Liu B, Mori I, Hossain MJ, Dong L, Takeda K, Kimura Y. Interleukin-18 improves the early defence system against influenza virus infection by augmenting natural killer cell-mediated cytotoxicity. J Gen Virol. 2004;85:423-428.

135. Liu L, Botos I, Wang Y, et al. Structural basis of toll-like receptor 3 signaling with double-stranded RNA. Science. 2008;320:379-381.

136. Liu S, Cai X, Wu J, et al. Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation. Science. 2015;347:aaa2630.

137. Liu S, Chen J, Cai X, et al. MAVS recruits multiple ubiquitin E3 ligases to activate antiviral signaling cascades. Elife. 2013;2:e00785.

138. Liu SY, Sanchez DJ, Aliyari R, Lu S, Cheng G. Systematic identification of type I and type II interferon-induced antiviral factors. Proc Natl Acad Sci U S A. 2012;109:4239-4244.

139. Liu X, Zhang Z, Ruan J, et al. Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. Nature. 2016;535:153-158.

140. Liu Y, Jesus AA, Marrero B, et al. Activated STING in a vascular and pulmonary syndrome. N Engl J Med. 2014;371:507-518.

141. Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. Annu Rev Immunol. 2005;23:275-306.

142. Lodoen M, Ogasawara K, Hamerman JA, et al. NKG2D-mediated natural killer cell protection against cytomegalovirus is impaired by viral gp40 modulation of retinoic acid early inducible 1 gene molecules. J Exp Med. 2003;197:1245-1253.

143. Lodoen MB, Abenes G, Umamoto S, Houchins JP, Liu F, Lanier LL. The cytomegalovirus m155 gene product subverts natural killer cell antiviral protection by disruption of H60-NKG2D interactions. J Exp Med. 2004;200:1075-1081.

144. Loo YM, Fornek J, Crochet N, et al. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. J Virol. 2008;82:335-345.

145. Lu A, Magupalli VG, Ruan J, et al. Unified polymerization mechanism for the assembly of ASC-dependent inflammasomes. Cell. 2014;156:1193-1206.

146. Lucas C, Wong P, Klein J, et al. Longitudinal analyses reveal immunological misfiring in severe COVID-19. Nature. 2020;584:463-469.

147. Luecke S, Holleufer A, Christensen MH, et al. cGAS is activated by DNA in a length-dependent manner. EMBO Rep. 2017;18:1707-1715.

148. Lund JM, Alexopoulou L, Sato A, et al. Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A. 2004;101:5598-5603.

149. Lund JM, Linehan MM, Iijima N, Iwasaki A. Cutting edge: plasmacytoid dendritic cells provide innate immune protection against mucosal viral infection in situ. J Immunol. 2006;177:7510-7514.

150. Majer O, Liu B, Kreuk LSM, Krogan N, Barton GM. UNC93B1 recruits syntenin-1 to dampen TLR7 signalling and prevent autoimmunity. Nature. 2019;575:366-370.

151. Majer O, Liu B, Woo BJ, Kreuk LSM, Van Dis E, Barton GM. Release from UNC93B1 reinforces the compartmentalized activation of select TLRs. Nature. 2019;575:371-374.

152. Malamut G, El Machhour R, Montcuquet N, et al. IL-15 triggers an antiapoptotic pathway in human intraepithelial lymphocytes that is a potential new target in celiac disease-associated inflammation and lymphomagenesis. J Clin Invest. 2010;120:2131-2143.

153. Mariathasan S, Weiss DS, Newton K, et al. Cryopyrin activates the inflammasome in response to toxins and ATP. Nature. 2006;440:228-232.

154. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell. 2002;10:417-426.

155. Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. Annu Rev Immunol. 2009;27:229-265.

156. Matyszewski M, Zheng W, Lueck J, et al. Distinct axial and lateral interactions within homologous filaments dictate the signaling specificity and order of the AIM2-ASC inflammasome. Nat Commun. 2021;12:2735.

157. Medzhitov R, Janeway CA Jr. Innate immune induction of the adaptive immune response. Cold Spring Harb Symp Quant Biol. 1999;64:429-435.

158. Merkl PE, Knipe DM. Role for a filamentous nuclear assembly of IFI16, DNA, and host factors in restriction of herpesviral infection. mBio. 2019;10:e02621-18.

159. Merkl PE, Orzalli MH, Knipe DM. Mechanisms of host IFI16, PML, and Daxx protein restriction of herpes simplex virus 1 replication. J Virol. 2018;92(10):e00057-18.

160. Mesev EV, LeDesma RA, Ploss A. Decoding type I and III interferon signalling during viral infection. Nat Microbiol. 2019;4:914-924.

161. Meylan E, Curran J, Hofmann K, et al. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature. 2005;437:1167-1172.

162. Moran I, Grootveld AK, Nguyen A, Phan TG. Subcapsular sinus macrophages: the seat of innate and adaptive memory in murine lymph nodes. Trends Immunol. 2019;40:35-48.

163. Morrone SR, Matyszewski M, Yu X, Delannoy M, Egelman EH, Sohn J. Assembly-driven activation of the AIM2 foreign-dsDNA sensor provides a polymerization template for downstream ASC. Nat Commun. 2015;6:7827.

164. Morrone SR, Wang T, Constantoulakis LM, Hooy RM, Delannoy MJ, Sohn J. Cooperative assembly of IFI16 filaments on dsDNA provides insights into host defense strategy. Proc Natl Acad Sci U S A. 2014;111:E62-E71.

165. Munoz-Planillo R, Kuffa P, Martinez-Colon G, Smith BL, Rajendiran TM, Nunez G. K(+) efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. Immunity. 2013;38:1142-1153.

166. Muruve DA, Petrilli V, Zaiss AK, et al. The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. Nature. 2008;452:103-107.

167. Myong S, Cui S, Cornish PV, et al. Cytosolic viral sensor RIG-I is a 5′-triphosphate-dependent translocase on double-stranded RNA. Science. 2009;323:1070-1074.

168. Neil SJ, Zang T, Bieniasz PD. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. Nature. 2008;451:425-430.

169. Nguyen KB, Salazar-Mather TP, Dalod MY, et al. Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. J Immunol. 2002;169:4279-4287.

170. Ohto U, Ishida H, Shibata T, Sato R, Miyake K, Shimizu T. Toll-like receptor 9 contains two DNA binding sites that function cooperatively to promote receptor dimerization and activation. Immunity. 2018;48:649-658 e644.

171. Ohto U, Shibata T, Tanji H, et al. Structural basis of CpG and inhibitory DNA recognition by Toll-like receptor 9. Nature. 2015;520:702-705.

172. Okamura H, Tsutsi H, Komatsu T, et al. Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature. 1995;378:88-91.

173. Oldenburg M, Kruger A, Ferstl R, et al. TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. Science. 2012;337:1111-1115.

174. Orzalli MH, Conwell SE, Berrios C, DeCaprio JA, Knipe DM. Nuclear interferon-inducible protein 16 promotes silencing of herpesviral and transfected DNA. Proc Natl Acad Sci U S A. 2013;110:E4492-E4501.

175. Orzalli MH, DeLuca NA, Knipe DM. Nuclear IFI16 induction of IRF-3 signaling during herpesviral infection and degradation of IFI16 by the viral ICP0 protein. Proc Natl Acad Sci U S A. 2012;109:E3008-E3017.

176. Park B, Brinkmann MM, Spooner E, Lee CC, Kim YM, Ploegh HL. Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. Nat Immunol. 2008;9:1407-1414.

177. Patel JR, Jain A, Chou YY, Baum A, Ha T, Garcia-Sastre A. ATPase-driven oligomerization of RIG-I on RNA allows optimal activation of type-I interferon. EMBO Rep. 2013;14:780-787.

178. Pathare GR, Decout A, Gluck S, et al. Structural mechanism of cGAS inhibition by the nucleosome. Nature. 2020;587:668-672.

179. Peisley A, Lin C, Wu B, et al. Cooperative assembly and dynamic disassembly of MDA5 filaments for viral dsRNA recognition. Proc Natl Acad Sci U S A. 2011;108:21010-21015.

180. Peisley A, Wu B, Xu H, Chen ZJ, Hur S. Structural basis for ubiquitin-mediated antiviral signal activation by RIG-I. Nature. 2014;509:110-114.

181. Peisley A, Wu B, Yao H, Walz T, Hur S. RIG-I forms signaling-competent filaments in an ATP-dependent, ubiquitin-independent manner. Mol Cell. 2013;51:573-583.

182. Petrilli V, Papin S, Dostert C, Mayor A, Martinon F, Tschopp J. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. Cell Death Differ. 2007;14:1583-1589.

183. Pichlmair A, Lassnig C, Eberle CA, et al. IFIT1 is an antiviral protein that recognizes 5′-triphosphate RNA. Nat Immunol. 2011;12:624-630.

184. Prokunina-Olsson L. Genetics of the human interferon lambda region. J Interferon Cytokine Res. 2019;39:599-608.

185. Ranoa DR, Parekh AD, Pitroda SP, et al. Cancer therapies activate RIG-I-like receptor pathway through endogenous non-coding RNAs. Oncotarget. 2016;7:26496-26515.

186. Raulet DH. Missing self recognition and self tolerance of natural killer (NK) cells. Semin Immunol. 2006;18:145-150.

187. Ren X, Linehan MM, Iwasaki A, Pyle AM. RIG-I Selectively discriminates against 5′-monophosphate RNA. Cell Rep. 2019;26:2019-2027 e2014.

188. Rice GI, Bond J, Asipu A, et al. Mutations involved in Aicardi-Goutieres syndrome implicate SAMHD1 as regulator of the innate immune response. Nat Genet. 2009;41:829-832.

189. Rice GI, Del Toro Duany Y, Jenkinson EM, et al. Gain-of-function mutations in IFIH1 cause a spectrum of human disease phenotypes associated with upregulated type I interferon signaling. Nat Genet. 2014;46:503-509.

190. Rice GI, Kasher PR, Forte GM, et al. Mutations in ADAR1 cause Aicardi-Goutieres syndrome associated with a type I interferon signature. Nat Genet. 2012;44:1243-1248.

191. Rivera-Serrano EE, Gizzi AS, Arnold JJ, Grove TL, Almo SC, Cameron CE. Viperin reveals its true function. Annu Rev Virol. 2020;7:421-446.

192. Roberts TL, Idris A, Dunn JA, et al. HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. Science. 2009;323(5917):1057-1060.

193. Roulois D, Loo Yau H, Singhania R, et al. DNA-demethylating agents target colorectal cancer cells by inducing viral mimicry by endogenous transcripts. Cell. 2015;162:961-973.

194. Sadler AJ, Williams BR. Structure and function of the protein kinase R. Curr Top Microbiol Immunol. 2007;316:253-292.

195. Saito T, Hirai R, Loo YM, et al. Regulation of innate antiviral defenses through a shared repressor domain in RIG-1 and LGP2. Proc Natl Acad Sci U S A. 2007;104:582.

196. Sasai M, Shingai M, Funami K, et al. NAK-associated protein 1 participates in both the TLR3 and the cytoplasmic pathways in type I IFN induction. J Immunol. 2006;177: 8676-8683.

197. Sato A, Linehan MM, Iwasaki A. Dual recognition of herpes simplex viruses by TLR2 and TLR9 in dendritic cells. Proc Natl Acad Sci U S A. 2006;103:17343-17348.

198. Satoh T, Kato H, Kumagai Y, et al. LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. Proc Natl Acad Sci U S A. 2010;107:1512-1517.

199. Schlee M, Roth A, Hornung V, et al. Recognition of 5′ triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. Immunity. 2009;31:25-34.

200. Schmitz N, Kurrer M, Bachmann MF, Kopf M. Interleukin-1 is responsible for acute lung immunopathology but increases survival of respiratory influenza virus infection. J Virol. 2005;79:6441-6448.

201. Schneider WM, Chevillotte MD, Rice CM. Interferon-stimulated genes: a complex web of host defenses. Annu Rev Immunol. 2014;32:513-545.

202. Schoggins JW. Interferon-stimulated genes: what do they all do? Annu Rev Virol. 2019;6:567-584.

203. Schoggins JW, Wilson SJ, Panis M, et al. A diverse range of gene products are effectors of the type I interferon antiviral response. Nature. 2011;472:481-485.

204. Schuberth-Wagner C, Ludwig J, Bruder AK, et al. A conserved histidine in the RNA sensor RIG-I controls immune tolerance to N1-2′ O-methylated self RNA. Immunity. 2015;43:41-51.

205. Schulz O, Diebold SS, Chen M, et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature. 2005;433:887.

206. Sen A, Feng N, Ettayebi K, Hardy ME, Greenberg HB. IRF3 inhibition by rotavirus NSP1 is host cell and virus strain dependent but independent of NSP1 proteasomal degradation. J Virol. 2009;83:10322-10335.

207. Seth RB, Sun L, Ea CK, Chen ZJ. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell. 2005;122:669-682.

208. Shang G, Zhang C, Chen ZJ, et al. Cryo-EM structures of STING reveal its mechanism of activation by cyclic GMP-AMP. Nature. 2019;567:389-393.

209. Sharif H, Wang L, Wang WL, et al. Structural mechanism for NEK7-licensed activation of NLRP3 inflammasome. Nature. 2019;570:338-343.

210. Shi J, Zhao Y, Wang K, et al. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature. 2015;526:660-665.

211. Shi Y, Yuan B, Qi N, et al. An autoinhibitory mechanism modulates MAVS activity in antiviral innate immune response. Nat Commun. 2015;6:7811.

212. Sims JE, Smith DE. The IL-1 family: regulators of immunity. Nat Rev Immunol. 2010;10:89-102.

213. Soveg FW, Schwerk J, Gokhale NS, et al. Endomembrane targeting of human OAS1 p46 augments antiviral activity. Elife. 2021;10:e71047.

214. Stetson DB, Ko JS, Heidmann T, Medzhitov R. Trex1 prevents cell-intrinsic initiation of autoimmunity. Cell. 2008;134:587-598.

215. Stetson DB, Medzhitov R. Type I interferons in host defense. Immunity. 2006;25:373.

216. Strahle L, Garcin D, Kolakofsky D. Sendai virus defective-interfering genomes and the activation of interferon-beta. Virology. 2006;351:101-111.

217. Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature. 2004;427:848-853.

218. Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science. 2013;339:786-791.

219. Swiecki M, Gilfillan S, Vermi W, Wang Y, Colonna M. Plasmacytoid dendritic cell ablation impacts early interferon responses and antiviral NK and CD8(+) T cell accrual. Immunity. 2010;33:955-966.

220. Szomolanyi-Tsuda E, Liang X, Welsh RM, Kurt-Jones EA, Finberg RW. Role for TLR2 in NK cell-mediated control of murine cytomegalovirus in vivo. J Virol. 2006;80:4286-4291.

221. Tabeta K, Hoebe K, Janssen EM, et al. The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. Nat Immunol. 2006;7:156-164.

222. Takahashi K, Kawai T, Kumar H, Sato S, Yonehara S, Akira S. Roles of caspase-8 and caspase-10 in innate immune responses to double-stranded RNA. J Immunol. 2006;176:4520-4524.

223. Takaoka A, Wang Z, Choi MK, et al. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature. 2007;448:501-505.

224. Takata MA, Goncalves-Carneiro D, Zang TM, et al. CG dinucleotide suppression enables antiviral defence targeting non-self RNA. Nature. 2017;550:124-127.

225. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol. 2003;21:335-376.

226. Takeda K, Tsutsui H, Yoshimoto T, et al. Defective NK cell activity and Th1 response in IL-18-deficient mice. Immunity. 1998;8:383-390.

227. Tanji H, Ohto U, Shibata T, Miyake K, Shimizu T. Structural reorganization of the Toll-like receptor 8 dimer induced by agonistic ligands. Science. 2013;339:1426-1429.

228. Thomas DL, Thio CL, Martin MP, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature. 2009;461:798-801.

229. Thomas PG, Dash P, Aldridge JR Jr, et al. The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. Immunity. 2009;30:566-575.

230. Ting JP, Lovering RC, Alnemri ES, et al. The NLR gene family: a standard nomenclature. Immunity. 2008;28:285-287.

231. Tomasec P, Braud VM, Rickards C, et al. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. Science. 2000;287:1031.

232. Toscano F, Estornes Y, Virard F, et al. Cleaved/associated TLR3 represents the primary form of the signaling receptor. J Immunol. 2013;190:764-773.

233. Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol. 2005;23:975-1028.

234. Unterholzner L, Keating SE, Baran M, et al. IFI16 is an innate immune sensor for intracellular DNA. Nat Immunol. 2010;11:997-1004.

235. Upton JW, Kaiser WJ, Mocarski ES. Virus inhibition of RIP3-dependent necrosis. Cell Host Microbe. 2010;7:302-313.

236. van der Veen AG, Maillard PV, Schmidt JM, et al. The RIG-I-like receptor LGP2 inhibits Dicer-dependent processing of long double-stranded RNA and blocks RNA interference in mammalian cells. EMBO J. 2018;37(4):e97479.

237. Veeranki S, Choubey D. Interferon-inducible p200-family protein IFI16, an innate immune sensor for cytosolic and nuclear double-stranded DNA: regulation of subcellular localization. Mol Immunol. 2012;49:567-571.

238. Venkataraman T, Valdes M, Elsby R, et al. Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses. J Immunol. 2007;178:6444-6455.

239. Versteeg GA, Garcia-Sastre A. Viral tricks to grid-lock the type I interferon system. Curr Opin Microbiol. 2010;13:508-516.

240. Volkman HE, Cambier S, Gray EE, Stetson DB. Tight nuclear tethering of cGAS is essential for preventing autoreactivity. Elife. 2019;8:e47491.

241. Weber F, Wagner V, Rasmussen SB, Hartmann R, Paludan SR. Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. J Virol. 2006;80:5059-5064.

242. Wickenhagen A, Sugrue E, Lytras S, et al. A prenylated dsRNA sensor protects against severe COVID-19. Science. 2021;374:eabj3624.

243. Wu B, Peisley A, Tetrault D, et al. Molecular imprinting as a signal activation mechanism of the viral RNA sensor RIG-I. Mol Cell. 2014;55:511-523.

244. Yang E, Li MMH. All about the RNA: interferon-stimulated genes that interfere with viral RNA processes. Front Immunol. 2020;11:605024.

245. Ye L, Schnepf D, Staeheli P. Interferon-lambda orchestrates innate and adaptive mucosal immune responses. Nat Rev Immunol. 2019;19:614-625.

246. Yoneyama M. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol. 2004;5:737.

247. Yoneyama M, Fujita T. Structural mechanism of RNA recognition by the RIG-I-like receptors. Immunity. 2008;29:178.

248. Yoshida R, Takaesu G, Yoshida H, et al. TRAF6 and MEKK1 play a pivotal role in the RIG-I-like helicase antiviral pathway. J Biol Chem. 2008;283:36211-36220.

249. Zeng W, Sun L, Jiang X, et al. Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity. Cell. 2010;141(2):315-330.

250. Zhang C, Shang G, Gui X, Zhang X, Bai XC, Chen ZJ. Structural basis of STING binding with and phosphorylation by TBK1. Nature. 2019;567:394-398.

251. Zhang SY, Jouanguy E, Ugolini S, et al. TLR3 deficiency in patients with herpes simplex encephalitis. Science. 2007;317:1522-1527.

252. Zhang X, Shi H, Wu J, et al. Cyclic GMP-AMP containing mixed phosphodiester linkages is an endogenous high-affinity ligand for STING. Mol Cell. 2013;51:226-235.

253. Zhang X, Wu J, Du F, et al. The cytosolic DNA sensor cGAS forms an oligomeric complex with DNA and undergoes switch-like conformational changes in the activation loop. Cell Rep. 2014;6:421-430.

254. Zhang Y, Xu Y, Dai Y, et al. Efficient Dicer processing of virus-derived double-stranded RNAs and its modulation by RIG-I-like receptor LGP2. PLoS Pathog. 2021;17:e1009790.

255. Zhang Z, Ohto U, Shibata T, et al. Structural analysis reveals that toll-like receptor 7 is a dual receptor for guanosine and single-stranded RNA. Immunity. 2016;45:737-748.

256. Zhang Z, Ohto U, Shibata T, et al. Structural analyses of toll-like receptor 7 reveal detailed RNA sequence specificity and recognition mechanism of agonistic ligands. Cell Rep. 2018;25:3371-3381 e3375.

257. Zhao B, Du F, Xu P, et al. A conserved PLPLRT/SD motif of STING mediates the recruitment and activation of TBK1. Nature. 2019;569:718-722.

258. Zhao B, Xu P, Rowlett CM, et al. The molecular basis of tight nuclear tethering and inactivation of cGAS. Nature. 2020;587:673-677.

259. Zhao X, Li J, Winkler CA, An P, Guo JT. IFITM genes, variants, and their roles in the control and pathogenesis of viral infections. Front Microbiol. 2018;9:3228.

260. Zheng M, Kanneganti TD. The regulation of the ZBP1-NLRP3 inflammasome and its implications in pyroptosis, apoptosis, and necroptosis (PANoptosis). Immunol Rev. 2020;297:26-38.

261. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. Nature. 2011;469:221-225.

262. Zhou S, Kurt-Jones EA, Mandell L, et al. MyD88 is critical for the development of innate and adaptive immunity during acute lymphocytic choriomeningitis virus infection. Eur J Immunol. 2005;35:822-830.

263. Zhu J, Martinez J, Huang X, Yang Y. Innate immunity against vaccinia virus is mediated by TLR2 and requires TLR-independent production of IFN-beta. Blood. 2007;109:619-625.

264. Zhu J, Zhang Y, Ghosh A, et al. Antiviral activity of human OASL protein is mediated by enhancing signaling of the RIG-I RNA sensor. Immunity. 2014;40:936-948.

265. Zilberstein A, Kimchi A, Schmidt A, Revel M. Isolation of two interferon-induced translational inhibitors: a protein kinase and an oligo-isoadenylate synthetase. Proc Natl Acad Sci U S A. 1978;75:4734-4738.

CHAPTER 10

The Adaptive Immune Response to Viruses

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Introduction

Overview of the adaptive immune response to virus infection

Development and architecture of the adaptive immune system

Generation of T and B lymphocytes: primary lymphoid organs

Secondary lymphoid organs: structure and function

Genes and molecules that mediate adaptive immunity

The CD1 family of class Ib genes and lipid recognition

T-cell receptor, coreceptor, and costimulatory genes and proteins

Antigen-presenting cells and the induction of adaptive immune responses

Dendritic cells activate naive T lymphocytes in secondary lymphoid organs

Activation of T and B lymphocytes in secondary lymphoid organs

Viral antigen recognition by B cells

Nature of B-cell epitopes

Antigenicity and immunogenicity

Generation of the primary B-cell repertoire

B-cell activation by viral antigens

Viral antigen recognition by T cells

Antigen processing and presentation to T cells

Antigen presentation by the major histocompatibility complex class I pathway

Antigen presentation by the major histocompatibility complex class II pathway

Induction of T-cell responses to antigen

Effector activities of B cells

Antiviral activities of antibody *in vitro*

Antiviral activities of antibody *in vivo*

Effector activities of T cells

$CD8^+$ effector mechanisms

$CD4^+$ T_E effector mechanisms

The phenomenon of $CD8^+$ T-cell exhaustion/dysfunction

Regulatory T-cell function in antiviral adaptive immune responses

Adaptive immune memory

B-cell memory

T-cell memory

Viral strategies to evade the adaptive immune response

Viral evasion of humoral (B-cell) immunity

Viral evasion of cellular (T-cell) immunity

Escape by subverting antigen processing and presentation

Interference of cytokine functions

Virus-induced immune dysregulation and autoimmune disease

INTRODUCTION

Viruses have been part of human ecology since the human species first evolved on the plains of East Africa. As humans moved out of Africa to populate the continental land masses, viruses, already adapted to the human species, were carried along with other microorganisms as part of the human ecological baggage. With the migration of humans around the world, we were exposed to new viruses present in the animal species that humans encountered. Some viruses, such as the herpes family members, Epstein-Barr virus (EBV), and herpes simplex virus (HSV) have co-evolved with humans and have developed complex mechanisms (eg, latency) to subvert the host defense mechanisms and sustain themselves in the human population. These viruses generally produce mild disease, except where host defenses are absent or weakened (eg, in immune-deficient or immune-suppressed individuals). Other human viruses (eg, measles virus) appear to have evolved mechanisms to persist at low levels in some infected individuals and sustain themselves by infecting previously unexposed individuals (typically children) who then go on to develop mild to severe illness and, in turn, serve as reservoirs for further virus propagation. Animal viruses can also *jump* from their host reservoir to infect humans and produce devastating diseases of potentially pandemic proportions. Three notable examples of zoonotic viral diseases are (1) the acquired immune deficiency syndrome (AIDS) pandemic produced by the human immunodeficiency virus (HIV), (2) the coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and (3) the periodic outbreaks of highly pathogenic avian influenza infection in poultry since 1997 in Asia and more recently in Africa and Europe, which have resulted in sporadic direct infections of humans with mortality rates approaching 50%. Indeed, the reconstruction and analysis of the genome of the influenza virus that caused the 1918 *Spanish flu* pandemic indicates that this virus was an avian influenza virus that spread directly to humans (without modification of virus in an intermediate host or the acquisition of genetic information from then-circulating human influenza strains).[193]

Virus infection can result in acute or chronic disease in virtually any organ and tissue of the body. Furthermore, infection with human papillomavirus (HPV), human herpesvirus 8 (HHV8), and hepatitis B (HBV) and C (HCV) viruses have been directly linked to the development of cervical cancer, Kaposi sarcoma, and hepatocellular carcinoma, respectively. As the devastating effects of HIV infection and the COVID-19 pandemic have taught us, virus infections are a continuing threat to human health. We can point to some notable successes in the struggle against viruses including the eradication of smallpox in the 1970s, the effectiveness of the live attenuated oral polio vaccine in eliminating paralytic polio in the developed world heralding the prospect of eradicating polio worldwide, and the successes of vaccines against SARS-CoV-2. These vaccination successes rely on the mobilization of the body's major defense against virus infection—the adaptive immune system. Our enthusiasm about these *successes* must be tempered, however. Because of the success of smallpox eradication, mandatory smallpox vaccination was discontinued in the United States in the early 1970s, making a large fraction of the population (ie, those under age 35 years) now susceptible to smallpox. This fact, along with the advances in poxvirus genetics and genetic engineering, now make smallpox a major bioterrorism threat. Similarly, administration of live oral polio vaccine to children in third world countries whose immune function is suppressed by malnutrition or HIV infection can result in outbreaks of paralytic polio caused by infection with virulent mutants (revertants) of the live attenuated vaccine strain. This realization has prompted a reevaluation of the prospect of global polio eradication using current vaccination strategies. Vaccines against SARS-CoV-2 protect against severe disease but cannot yet protect us from infection by antigenically altered viral variants. Strain-specific vaccines that minimally cross-react antigenically with previously employed vaccines, or pan-coronavirus vaccines, might be necessary. To counter the continuing threat posed by viruses, it is essential to understand the body's primary defense against virus infection in the immune system.

Overview of the Adaptive Immune Response to Virus Infection

Viruses are obligatory intracellular parasites; that is, they replicate within cells of the infected host and utilize the cell's biosynthetic machinery for replication and production of progeny virions. Furthermore, most viruses replicate rapidly in infected cells (ie, within hours) producing progeny virions capable of infecting additional cells, thereby propagating and sustaining infection in the host. These properties of infection by viruses dictate how the immune system must respond to counter infection. In acute virus infection, the race between the replication of the virus and the host response will result either in the death of the host or in virus clearance and the termination of infection. The tissue injury produced by viruses at the site(s) of infection results both from the cytopathic effects of virus replication and, equally importantly, from the host immune response to infection. In general, therefore, the greater the extent of virus replication in the infected host, the greater the tissue injury and illness severity.

In chronic infection, the time scale of virus replication in the host is not measured in days (as in acute infection) but in weeks, months, or even years, with the host immune response likewise continuing over the same prolonged time scale. Viruses that produce chronic, persistent infection have evolved mechanisms to suppress or alter the immune response (allowing these viruses to persist in the infected host). The tissue injury produced by the immune response to virus infection (termed, *immunopathology*) occurs with both acute and chronic virus infection; in many chronic virus infections (eg, HCV infection), however, immune-mediated tissue injury predominates (rather than direct virus-mediated injury).[96] Furthermore, the clinical manifestations of virus infection (eg, fever, headache, myalgia, anorexia) are primarily caused by inflammatory mediators (eg, cytokines) released by cells of the immune system in response to infection. Finally, the triggering of the immune system by virus infection can result in the induction of aberrant immune responses directed both to the virus and to self-cellular constituents, resulting in autoimmune disease.[16,108]

The primary host defenses against virus infection are physical/chemical barriers to infection and the immune system. The immune system can be divided into two components: the innate immune system and the adaptive immune system. This division is based on the properties of the immune cell types and molecules involved in the response to infection and the tempo of the response (Table 10.1). The response of the innate immune system to virus infection is either immediate (ie, constitutively active) or rapidly induced (ie, within hours of infection). The innate immune response is triggered in two ways: first, through recognition of viral constituents (eg, viral nucleic acids) by a limited set of cellular pattern recognition receptors for foreign molecules displayed on innate immune cells and, second, through the activation of intracellular signaling mechanisms following virus entry into cells and the initiation of virus replication. Importantly, a defining feature of the innate immune

response is that repeat exposure to a particular virus generally evokes an identical response from the innate immune system, although some epigenetic "memory" may partly strengthen some responses.

TABLE 10.1 Cells of the immune system

Type	Subtype	Onset of Effect
Innate immune cells		
Granulocytic	Neutrophils, basophils, eosinophils	Immediate/ inducible (h)
Monocytic	Macrophage, DC	Immediate/ inducible (h)
Lymphocytic[a]	Innate lymphoid cells, NK cells, NK T cells	Immediate/ inducible (h)
Somatic cell (nonhematopoietic)	Epithelial, endothelial cells, etc.	Immediate/ inducible (h)
Adaptive immune cell		
B lymphocyte	—	Inducible (d)
T lymphocyte	$CD4^+$, $CD8^+$, T_{reg}	Inducible (d)

[a]Specific subtypes of T lymphocytes (eg, γδ T cells and intraepithelial [IEL] $CD8^+\alpha\alpha$ T lymphocytes) are considered to be part of the innate immune system. These cell types with lymphocytic morphology employ a TCR-type antigen recognition system but differ in this function and strategy of antigen recognition from conventional T cells. Plasmacytoid dendritic cells are now considered to be of lymphoid origin.
DC, dendritic cells; NK, natural killer; h, hours; d, days.

By contrast, the response of the adaptive immune system to a first encounter with a virus takes days to evolve. The response of the cells of the adaptive immune system (ie, B lymphocytes and T lymphocytes) is triggered by viral constituents, through the engagement of highly specific cell surface recognition receptors displayed by a minuscule fraction of the total repertoire of adaptive immune cells for any given virus. These receptors only recognize the constituents (antigens) of that particular virus. Importantly, this arm of the immune system *adapts* to repeat exposure to a particular virus or viral protein; this results in a more rapid response of higher magnitude by the specific adaptive immune cells on repeat exposure/infection—a phenomenon called *immunological memory*, the basis for vaccination.[2,51,74,134,147,152,157,179]

Circulating molecules of the innate immune response include proteins of the complement system and circulating agglutinins, including pentraxins and lectins of the collectin and ficolin families. These molecules are present in the blood and tissue fluids and can bind or be covalently attached (in the case of complement proteins) to certain viruses, thereby inhibiting virus infection. Leukocytes, in particular, neutrophils, blood monocytes, tissue macrophages, dendritic cells (DCs), and innate lymphoid cells including natural killer (NK) cells are the dominant, innate immune cell types that respond to virus infection. They are triggered/activated through the engagement of pattern recognition receptors (eg, Toll-like receptors [TLR]) expressed on the cell surface, in endosomes, or in the cytosol, that recognize viral constituents (eg, viral nucleic acids). Some of these cells reside in the tissues, and they respond there while others are recruited to sites of infection from the bloodstream in response to cytokines and chemokines released during infection.

Nonhematopoietic body cells (eg, epithelial cells, endothelial cells, fibroblasts) that are targets of virus infection must also be considered part of the innate immune response because, on infection, they are triggered to secrete antiviral proinflammatory mediators such as type 1 interferons and certain cytokines/chemokines (the production of which can initiate and amplify the innate immune response to infection). The induction of the innate immune response and the role of innate immune cells and molecules in controlling virus infection are discussed in detail elsewhere (Chapter 8). One of these innate immune cell types, the conventional DC, however, bridges the innate and adaptive immune response, and its role in the initiation of the adaptive immune response is discussed in a later section of this chapter.

A hallmark of the adaptive immune response is the exquisite specificity of adaptive immune cells for a particular foreign viral antigen and the capacity of this system to recognize and respond to a myriad of viruses (or other antigens). This remarkable property of the adaptive immune cells is achieved by the generation of a correspondingly diverse array of cell surface recognition receptors, which are randomly generated within the lymphocytes of every individual and clonally distributed among the adaptive immune cells. As a consequence, in a nonimmune individual, only an extremely small fraction of the adaptive immune cell repertoire (eg, one cell in a million) may have an antigen receptor capable of recognizing a specific viral antigen. Understanding how this diverse array of clonally distributed antigen receptors is generated during lymphocyte development and how rare adaptive immune cells specific for particular viral antigens find infected cells in a peripheral site of infection (eg, the skin or lungs) is critical to the understanding of the adaptive immune response to virus infection.

Viruses, because they exist as extracellular virion particles and replicate/assemble within infected cells, pose a unique recognition problem for the adaptive immune system (ie, how to recognize the virus [viral proteins or genome] both outside of the cell and within the infected cell). The elegant solution to this problem employed by the adaptive immune system is the partitioning of the adaptive immune system into two distinct cell types: B and T lymphocytes (Table 10.2). B lymphocytes displaying a receptor specific for a particular virus will, on encounter with the virus and activation, release their antigen receptor in a soluble form (ie, as antibody molecules) capable of binding free (extracellular) virions in a highly specific matter. This interaction typically results in virus neutralization/elimination. Antiviral B lymphocyte responses are exquisitely sensitive to the three-dimensional confirmation of the virus (and its constituents), and they play a dominant role in clearing virus during infection, in preventing or limiting reinfection (after previous infection with a virus), and in vaccination against a specific virus.

TABLE 10.2 Properties of T and B lymphocytes

General Properties	B Lymphocytes	T Lymphocytes
Origin	Bone marrow	Bone marrow
Self/ non–self-discrimination	Bone marrow and periphery	Thymus and periphery
Activation	BCR and other ligand receptors	TCR and other ligand receptors
Antigen recognition	Native (3-D) conformation	Nonnative fragments
Antigen receptor	**B Lymphocytes**	**T Lymphocytes**
Somatically generated	+	+
High specificity	+	+
Clonally represented	+	+
Localization	Cell surface/ secreted (Ab)	Cell surface only

BCR, B-cell receptor; TCR, T-cell receptor; Ab, antibody.

Activated antiviral T lymphocytes retain their antigen-specific receptors on the cell surface and are responsible for recognizing and eliminating virus-infected cells. T lymphocytes, however, do not recognize viral proteins in their native conformation. Rather, the antigen receptors on the T lymphocytes recognize *processed* peptide fragments of viral proteins displayed on the infected cell surface bound to cell surface molecular recognition platforms (ie, the major histocompatibility complex [MHC] locus gene products). Activated virus-specific T lymphocytes halt the spread of infection through direct cell-to-cell contact with virally infected cells, which may then be killed by these T cells. They also contribute to clearance or containment by the release of soluble mediators that include cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor (TNF). By releasing cytokines and chemokines, activated T lymphocytes can recruit and orchestrate the activation of innate immune cells, which can also facilitate viral clearance. Understanding how and where B and T lymphocytes are activated, the mechanism of antigen presentation to these cells, and the range of effector activities that these cells use to control and eliminate virus is essential for understanding the adaptive immune response to virus infection. These important topics are reviewed in this chapter.

Finally, the importance of the immune system in general and the adaptive immune response in particular in controlling virus clearance and promoting recovery from infection is exemplified by the fact that viruses have evolved a variety of mechanisms to inhibit or alter the host immune response. Whereas these viral immune evasion strategies are best considered in the context of viral pathogenesis (Chapter 11) and are addressed in chapters dealing with individual viruses, this chapter highlights some of the viral strategies that contribute to the evasion of adaptive immune responses.

Development and Architecture of the Adaptive Immune System

Generation of T and B lymphocytes: Primary Lymphoid Organs

Unlike the cells of the innate immune system, T and B lymphocytes possess one unique and profoundly important property. On their surfaces, these cells also display a receptor complex capable of recognizing foreign (nonself) structures—with exquisite specificity. The extraordinary diversity of adaptive immune cells is generated by a process of somatic recombination of antigen receptor gene segments. These gene segments are randomly selected and joined together in individual developing lymphocytes. The recombination-based creation, in each developing lymphocyte, of a different antigen receptor contributes to tremendous clonal diversity. Thus, of the ~10^9 naive T and B lymphocytes in the human adaptive immune system, each cell may, in principle, display a unique receptor directed to a specific foreign antigenic structure. This common property of the antigen receptor on T and B lymphocytes (ie, random generation by somatic DNA recombination and clonal receptor display) immediately raises some important questions. If these antigen receptors are stochastically generated, how is the generation of lymphocytes with receptors to self-structures (eg, tissue proteins) avoided? And if the frequency of lymphocytes with a receptor directed to a specific foreign antigen (eg, the coat proteins of an invading virus) is extremely low (1 in 10^7 lymphocytes), how can those rare lymphocytes locate the antigen quickly enough to respond before the infection is beyond control? As will be discussed below, it is the unique structure of the primary (central) lymphoid organs where lymphocyte progenitors develop into mature T and B lymphocytes, and of the secondary (peripheral) lymphoid organs where mature T and B lymphocytes respond to invading microorganisms, that provide some of the answers to these questions.

Mature T and B lymphocytes are derived from lymphoid progenitors that emerge prenatally in the fetal liver and subsequently, after birth, in the bone marrow, through a process of differentiation and selection linked to, and driven by, the antigen receptors displayed by these cells.[2,51,74,134,147,152,157,179] As outlined in Table 10.2, B lymphocyte development occurs after birth in the bone marrow (and prenatally in the fetal liver) and follows a well-defined sequential program. Initially lineage-defining transcription factors commit common lymphoid progenitors to the B lineage and induce the expression of genes whose products initiate the sequential rearrangement of immunoglobulin (Ig) D_H and J_H, heavy chain genetic elements (by random somatic recombination), at the pro-B stage. This is followed by V_H to DJ_H rearrangement. Combinatorial rearrangements between about 45 V_H segments, 23 J_H segments, and 6 J_H segments contribute to a different fusion VDJ exon in each developing B cell that encodes the V domain of an Ig heavy chain protein.[2] Downstream C region exons encode the Ig heavy chain constant domains. IgH gene rearrangement, transcription, RNA splicing, and protein expression are followed by a checkpoint at the transition between the pro-B and the large pre-B stages that ensures that only developing pre-B cells with productive IgH gene rearrangements capable of yielding a complete IgH protein (initially the IgM μ protein) are preserved; this checkpoint also ensures allelic exclusion—the process that maintains clonal

specificity by ensuring that only one (maternal or paternal) IgH locus is productively rearranged in each developing B cell. This is followed, at the small pre-B stage, by rearrangement of the Ig light chain kappa locus (or later the lambda locus) involving V_L to J_L rearrangements, thus yielding diverse "immature" B cells in the bone marrow, each now expressing a surface IgM molecule that serves as the B-cell receptor. A large proportion of these surface IgM-expressing immature B cells in the bone marrow are specific for self-molecules (ie, proteins, lipids, carbohydrate, nucleic acid) and undergo a process of negative selection called receptor editing that involves replacement of the existing rearranged light chain gene with a new V_L to J_L rearrangement (first at the kappa locus, and if that fails, at the lambda Ig light chain locus), that yields a non–self-reactive BCR. B cells that do not bind to self-antigens leave the bone marrow as transitional B cells and eventually seed peripheral lymphoid organs as naive follicular B cells. At the transitional B-cell stage and beyond, self-reactive B cells can still be tolerized by an apoptotic clonal deletional process or by a process of induced dysfunction called anergy.

The rearranged IgH gene produces two types of related transcripts; one encodes an Ig H-chain protein with a transmembrane anchor that associates with a L-chain to form the BCR while an alternatively spliced version that is shorter, encodes the Ig H-chain of soluble, secreted antibodies. The membrane-bound Ig H-L tetramer (the BCR) is transported from the endoplasmic reticulum (ER) to the cell surface by two proteins that associate with it called Igα and Igβ; these proteins are also essential for BCR signal transduction and for the endocytic internalization by the BCR of specific antigens. The ability of each B cell to express a surface BCR initially and to later, after activation, secrete antibodies that bind the same antigen as the original BCR, has two important implications for the recognition of viruses by B lymphocytes: (1) the B lymphocyte response to viruses will be particularly effective when interacting with free/extracellular virus constituents (eg, virions) and (2) the BCR and the secreted antibody generated after B-cell activation will be sensitive to the conformation of the viral constituent recognized.

T lymphocyte lineage development follows a program similar to that of B lymphocytes. The T-cell antigen receptor (TCR) is also a heterodimer consisting of two polypeptide chains, each of which is formed by somatic recombination of variable (V), diversity (D), and joining (J) segments encoding the variable portion of one chain (or V and J joining for the other chain). This process generates a VDJ exon or a VJ exon encoding the variable (V) domain of each TCR polypeptide, while a downstream unrearranged exon contributes to the constant (C) domain. Like the BCR, the TCR is an integral membrane protein but no alternative secreted antibodylike form of the TCR exists in nature. Unlike the BCR, the TCR does not recognize intact or fragmented microbial products directly but rather recognizes small fragments of molecules—typically, short peptide fragments of microbial or self-proteins bound to and exhibited on cell surfaces by protein products of the mammalian MHC genetic locus (eg, the classically defined major human transplantation antigens). These cell surface MHC gene products serve as molecular platforms, both selecting and displaying the self-peptides involved in TCR selection during T lymphocyte development in the thymus and also presenting microbial peptides to mature T lymphocytes that respond to microbial entry or infection of host cells. Consequently, T lymphocytes are uniquely suited to recognize host cells that harbor microorganisms (eg, viruses and intracellular bacteria). The steps in the processing (proteolytic fragmentation) and presentation of both self and microbial gene products by MHC locus proteins, and the structure of the major classes of MHC proteins (ie, MHC class I and II proteins), are discussed below.

T lymphocyte development occurs primarily in the thymus (Fig. 10.1). Lymphoid progenitors from the bone marrow enter the thymus from the blood, where they differentiate into T lymphocytes that then leave the thymus, enter the circulation, and ultimately populate secondary lymphoid organs (SLOs). Two classes of mature T lymphocytes are produced in the thymus. The major population (~80% of the peripheral T lymphocyte pool) are αβ T cells (so-called because their heterodimeric TCR express two chains encoded by TCR-α and β loci). The second minor population of mature T lymphocytes is called γδ T cells (because they express a related two-chain TCR derived from the TCR-γ and δ loci). Much of our current information on T lymphocyte development comes from analysis of αβ T-cell development. These T lymphocytes represent the T-cell subset that primarily responds to viral infection, and the selection process of these T cells in the thymus will be emphasized here. The γδ T-cell subset likely plays a limited role in the adaptive response to virus infection, and the properties and functions of this subset are summarized below.

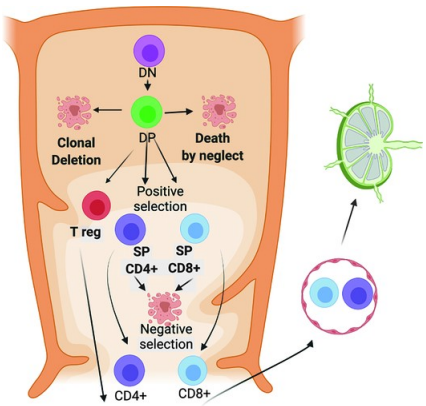

FIGURE 10.1 **T lymphocyte development and selection in the thymus**. In the thymic cortex, $CD4^-/CD8^-$ double-negative (*DN*) thymocytes mature into $CD4^+$ $CD8^+$ double-positive (*DP*) thymocytes. DP thymocytes with T-cell antigen receptors that interact with moderate avidity for self-MHC (major histocompatibility complex) class II or class I molecules presenting self-peptides are, respectively, *positively selected* into $CD4^+$ or $CD8^+$ single-positive (*SP*) thymocytes, which migrate into the thymic medulla. DP thymocytes that fail to bind self-MHC die by apoptosis (death by neglect), while those that bind very tightly to self-MHC-peptide complexes in the cortex are *negatively selected* (marked in the figure as clonal deletion). Positively selected single-positive thymocytes undergo further clonal deletion in the thymic medulla and surviving single-positive $CD4^+$ and $CD8^+$ T cells as well as regulatory T cells (Treg) egress the thymus into the bloodstream. Naive T cells home to secondary lymphoid organs and recirculate.

T lymphocyte development proceeds in a stepwise fashion with the sequential gene rearrangement and expression of one (ie, TCR-β or TCR-δ), then the other (ie, TCR-α or TCR-γ TCR chain) TCR genes as thymocytes mature. For TCR-β chains, one of ~50 V_β gene segments recombines with a single diversity (D_β) segment and one of 6-7 J_β gene segments to give rise to the rearranged $V_\beta D_\beta J_\beta$ exon encoding the V domain of the TCR-β chain (the D, J, and C region of the TCR-β locus in humans is duplicated and rearrangement occurs randomly in one of these duplicated regions in any given developing T cell). For human TCR-α chain formation, one of ~45 distinct V_α genes recombines with one of ~55 joining (J_α)

gene segments and the associated downstream C gene segment to produce the rearranged gene $V_\alpha J_\alpha$ exon encoding the V domain of the TCR-α chain. Further, TCR diversity is generated by the enzymatic addition/deletion of nucleotides during and e V_β-D_β-J_β and V_α-J_α recombination. The TCR on γδ T cells is generated by an identical process involving the rearrangement of V_γ-J_γ and V_δ-D_δ-J_δ segments. Four cell surface molecules including three CD3 proteins (CD3 γ, δ, ε) and the ξ protein noncovalently associate with the TCR (analogous to Igα and Igβ for the BCR), transport it to the cell surface and are critical for signaling through the TCR. Each TCR dimer associates, initially in the ER membrane with a CD3 γε heterodimer, a CD3 δε heterodimer, and a ξξ homodimer, with a total of eight polypeptides making up the TCR complex. This complex must form for the TCR to be transported to the plasma membrane. Specific antigen recognition by the TCR-αβ (or γδ) heterodimer results in signaling that is dependent on the CD3 and ξ components of the TCR complex.

Because the TCR does not recognize foreign molecules directly (but rather small fragments of proteins bound to MHC molecules), the TCR generated and expressed on developing thymocytes must recognize both MHC molecules and the peptide fragments bound to these molecules. The source of these peptides bound by MHC class I and class II molecules in the thymus are self-proteins expressed by several different cell types in the thymus. The selection of the thymocytes that ultimately become mature *single-positive* T lymphocytes ($CD4^+$ or $CD8^+$) and populate the SLOs is a two-step process in the thymus: first positive selection followed by negative selection.

The most immature T-cell progenitors commit to the T lineage and differentiate after being triggered by Notch ligands on thymic cortical epithelial cells (cTECs). Initially, they do not express the CD4 or CD8 coreceptor proteins and are therefore called *double-negative* (DN) thymocytes. These cTECs also provide the IL-7 cytokine that supports the proliferation and survival of DN thymocytes. DN thymocytes begin to rearrange their T-cell receptor genes in an ordered fashion. In the case of αβ T cells, TCR Dβ to Jβ gene rearrangement is followed by Vβ to DJβ rearrangement and a checkpoint ensues whereby signaling ensures that only cells able to express an intact TCR-β chain protein are selected, and allelic exclusion at this checkpoint ensures that each T-cell clone will eventually only express one TCR-β chain protein. Selected cells then make the TCR-α locus accessible to randomly recombine TCR Vα and Jα gene segments. As a result, T cells with a complete αβ TCR and that now simultaneously express both the CD4 and CD8 coreceptors emerge. These *double-positive* (DP) T cells are still in the thymic cortex where they undergo positive selection by self-MHC and peptide presented on cTECs.

The "purpose" of positive selection is to assign to each T-cell the coreceptor that matches the class of self-MHC molecules that its TCR recognizes. There is an intrinsic structural bias of all αβ TCRs to recognize MHC molecules, and a very small fraction of DP T cells have randomly generated TCRs that are unable to bind to self-peptide/MHC molecular complexes, and they are not positively selected. In the absence of a signal delivered through the TCR, these thymocytes will die by apoptosis (a process called "death by neglect" for these cells). Those DP thymocytes whose TCRs can bind specific self-peptide/MHC complexes above a threshold avidity, augmented by either a CD4 or a CD8 coreceptor binding to the MHC molecule, will undergo positive selection and survive. If the DP thymocyte TCR binds a peptide bound to MHC class II, this interaction results in the silencing of the CD8 coreceptor gene and preservation of CD4. This signaling event thus results in the development of a $CD4^+$ single-positive thymocyte whose TCR recognizes (is restricted to) peptides bound to MHC class II molecules. Similarly, the DP thymocytes whose TCRs recognize MHC class I-self-peptide complexes with sufficient avidity to survive (ie, those that are positively selected) will give rise to MHC class I–restricted $CD8^+$ single-positive thymocytes by a broadly similar silencing mechanism but of the CD4 gene. Although in the cortex the major selection event is positive selection, the recognition by any TCR of MHC-self-peptide complexes on cTECs with very high avidity results in apoptotic death or negative selection of those developing T cells. However, negative selection also occurs in the thymic medulla at the single-positive (SP) thymocyte stage.

After undergoing positive selection, the surviving SP thymocytes migrate into the medulla. They are all intrinsically autoreactive (ie, directed to self-peptide/MHC complexes), but these cells had not encountered self-MHC-peptide complexes of really high avidity in the thymic cortex. A process of negative selection must ensue to eliminate self-reactive SP T cells that can recognize self-peptides, especially from different part of the body, presented on self-MHC molecules and that have high avidity for TCRs on developing T cells. Failure to eliminate or eventually suppress such strongly self-reactive T cells could result in autoimmunity. Most negative selection in the medulla is mediated either by circulating professional APC, particularly plasmacytoid DCs (pDCs) and conventional DCs (DCs), or by medullary thymic epithelial cells (mTECs). Both pDCs and DCs can capture self-proteins that originate in different organs and tissues and enter the circulation either as soluble proteins or shed in exosomes. Some of these proteins will be processed in late endosomes, and resultant peptides are presented on MHC class II molecules, while other proteins are processed in proteasomes and peptides so derived are presented on MHC class I molecule (as discussed later in this chapter). Consequently, when SP thymocytes encounter these self-peptide/MHC complexes displayed by professional APC in the medulla with high avidity, these T cells will be eliminated (ie, undergo negative selection—death by apoptosis).

Another very important process of negative selection that is mediated by mTECs occurs in the thymic medulla. Individual mTECs turn on entire batteries of genes that characterize specific peripheral cells. This differentiation of mTECs into partial phenocopies of many different types of peripheral cells is dependent on a protein called AIRE that is mutated in a rare single gene human autoimmune disorder.[14,130] This differentiation process allows the expression, processing, and presentation by mTECs of peptides that are representative of tissue-specific peripheral proteins expressed across many different tissues and cell types. As a result, many self-reactive SP T cells with TCRs that have a high avidity for a wide range of self-peptides are deleted in the medulla.

Only about 2%-5% of immature thymocytes will survive the positive and negative selection processes. These remaining thymocytes emerge as mature naive $CD4^+$ or $CD8^+$ T lymphocytes with TCRs that recognize peptide/MHC complexes but will only interact with the MHC molecules with sufficient avidity to be activated when the appropriate foreign (microbial) peptides are bound to the MHC molecule. These naive single-positive αβ T lymphocytes will leave the thymus to populate SLOs and recirculate (Fig. 10.1). $CD4^+$ and $CD8^+$ αβ T cells represent 70%-80% of the circulating extrathymic T-cell population—they are the primary T cells that respond to virus infection.

The second (minor) population of thymus-derived cells that populate peripheral sites are the γδ T cells, which are so named because they employ a heterodimeric TCR whose variable (and constant) region are encoded by a distinct set of genetic loci (the γ locus and the δ locus) homologous to the α and β TCR loci[2,51,74,134,147,152,157,179] (Table 10.3). They are sometimes referred to as innatelike T cells. These TCR genes rearrange in the thymus in a manner analogous to that of the dominant αβ TCR T-cell homolog. The generation of γδ T cells in the thymus precedes αβ T-cell development. The γδ T cells are generated in two waves during T-cell development in the fetus. The first wave of maturing thymocytes consists of cells with limited TCR V gene diversity (ie, utilize one or few Vγ and Vδ rearrangement in their TCR). After selection, these γδ T cells leave the thymus, traffic through the circulation, and preferentially localize to epithelial surfaces, particularly the skin, gut, and urogenital tract. The second wave of γδ T cells, as with αβ T cells, displays a highly diverse TCR array, and these cells localize to the secondary lymphoid tissues. Unlike the αβ TCR, however, the γδ TCR does not recognize peptides bound to the MHC class I or II proteins. These γδ T cells either directly recognize structures such as heat shock proteins displayed on the surface of the infected cells or recognize lipids or

phosphorylated metabolites bound to MHC-like molecules (eg, nonpolymorphic MHC Ib molecules) on the surface of stressed (infected) cells. Intraepithelial γδ T cells function like cells of the innate immune system, recognizing not a specific pathogen but alterations in epithelial cells induced by infection with microbes such as bacteria. At present, no direct role for γδ T cells in the control of virus infection has been established.

TABLE 10.3 T lymphocyte subsets

αβ T	Dominant T-Cell–Type Responding to Virus Infection		
	$CD8^+$	Recognize major histocompatibility complex (MHC) class I viral peptide complexes, and are important adaptive immune antiviral effector cells	
	$CD4^+$	Recognize MHC class II viral peptide complexes, and regulate B-cell differentiation and host inflammatory responses to infection	
		T_H1	Effector $CD4^+$ T cells that produce IFN-γ
		T_H17	Effector $CD4^+$ T cells that secrete IL-17 and IL-22
		T_H2	Effector $CD4^+$ T cells that produce IL-4, IL-5, and IL-13
γδ T	Play a prominent role as "innatelike" effector cells • Express the CD3 complex, but usually not CD4 or CD8 • The generation process of γ:δ TCR chains in the thymus is similar to that of the dominant TCR-α:β T-cell TCR • Present in the circulation and lymphoid organs and prominently expressed at body surfaces (eg, gut, skin) • Recognize products of stressed cells—no restriction in recognition by major histocompatibility complex (MHC) class I/II molecules but can recognize lipid complexed to MHC class Ib molecules		
NKT	Act as "innatelike" immune effectors early in immune responses • Express conventional TCR-αβ chains, but use a restricted number of possible TCR genes • Express CD4 molecules (most NK T cells) and NK cell markers but not CD8 molecules • Recognize products of stressed cells and can recognize lipid moieties displayed by MHC class Ib–related molecules on cell surfaces • Activation of TCR triggers release of regulatory cytokines (eg, IL-4)		
T_{reg}	Conventional TCR-αβ T cells that control the response of T and B lymphocytes to foreign (eg, viral) antigens and self-proteins • Dominant population expresses CD4 and CD25 • May occur spontaneously during T-cell differentiation or after exposure to foreign (eg, viral) antigen • Regulates (usually suppresses) the response of $CD4^+$ T cells and $CD8^+$ T cells to infectious agents and self-constituents		

IFN, interferon; TCR, T-cell receptor; IL, interleukin; NKT, natural killer T cells.

Among the other T lymphocyte subsets (Table 10.3) generated during development are invariant NK T cells, so-called because these αβ TCR–expressing T cells display cell surface markers shared with NK cells and, similar to γδ T cells, they utilize a limited number of TCR V genes in receptor generation. Other NK T cells exist that are not invariant, but all NK T cells recognize lipid antigens presented on a family of MHC class I–like molecules called CD1 molecules. They are also, like γδ T cells, sometimes considered to be innatelike T cells. Another type of innatelike T-cell population with an invariant TCR, called mucosa-associated invariant T cells or MAIT cells, recognize fungal or bacterial metabolites derived from the riboflavin synthesis pathway that presented on an MHC class I–related protein (MR1). MAIT cells are abundant in the gut and liver, express CD8, secrete cytokines upon encountering infected cells including virally infected cells, and have been implicated in controlling some viral infections.

During T lymphocyte development, the thymus also generates cells called regulatory T cells or (Tregs), a subset of $CD4^+$ T cells that are critical regulators of adaptive immune responses to infectious agents, self-molecules, and allergens. These Tregs are often called "natural" or "thymic" Tregs to distinguish them from very similar cells that are generated in the periphery from mature $CD4^+$ T cells. Tregs suppress the activity of conventional $CD4^+$ and $CD8^+$ T cells responding to viral infection. They prevent the development of excessive injury during T-cell responses to infection and suppress the development of autoimmune responses during viral infection.

Secondary Lymphoid Organs: Structure and Function

Mature naive (resting) B and T lymphocytes egress central lymphoid organs (the bone marrow and thymus, respectively) and acquire the ability to recirculate through the blood to and from SLOs. The major SLOs are the lymph nodes (LN), the spleen, and the so-called mucosal-associated lymphoid tissue (MALT). Recirculation of naive B and T cells refers to the homing of naive (and some categories of memory) lymphocytes to SLOs through the blood, then egress into efferent lymphatics that drain into the thoracic duct, and then reentry into the blood for the next homing event. This recirculatory process continues throughout the lifespan of each naive lymphocyte, unless this cell encounters a specific antigen in an SLO and is then activated.

SLOs play a critical role in the initiation of the adaptive (T- and B-cell) immune response to viruses and other microbes. LN are highly organized structures located at vessel convergence points in the lymphatic system, a system that collects fluids and cells from the tissues of most internal organs (eg, liver, stomach) and body surfaces (eg, skin, mucosa of the gastrointestinal [GI], genitourinary [GU], and respiratory tracts). The capacity of lymphatic vessels to collect antigen (eg, virus, viral constituents) from the body surfaces/tissues and deliver them to the LN (where antigen is concentrated) is essential for the efficient triggering of the adaptive immune response to microbes. The spleen plays a similar role as the LN network; however, the spleen is uniquely suited to collect antigen (virus) directly from the bloodstream, because of its unique architecture and extensive blood circulation. The third class of SLO, the MALT, is composed of specialized lymphoid aggregates associated with major mucosal surfaces and sites of microbial invasions (eg, the GI, GU, and respiratory tracts). The gut-associated MALT (also called GALT) includes the tonsils, adenoids, appendix, and Peyer patches in the small intestine. In the respiratory tract, there are similar but more diffuse aggregates of lymphoid tissues associated with the epithelium of small/medium airways (ie, bronchial-associated lymphoid tissue [BALT]). As with the other SLO, the MALT are sites of antigen localization where the initiation of the adaptive immune response occurs.

The common feature of SLO is the localization of adaptive immune cells (ie, T and B cells) to distinct regions of the SLO. For example, most B cells are localized in distinct follicles within the LN cortex. Activation of B cells specific for a pathogen/antigen by protein antigens and with T-cell help can lead to their proliferation and formation of germinal centers (GCs) within the LN cortex. T cells within the LN localize to, and are diffusely distributed within, the LN paracortical areas. Other cell types are also distributed within the outer cortex and central medulla of the normal LN. These include stromal cells and cells of macrophage and DC lineage. These cells (particularly DC) play a crucial role in the induction of the adaptive immune response, and some stromal cells serve to direct T-cell/B-cell localization to distinct LN regions through the constitutive release of small (low molecular weight) chemotactic proteins or chemokines. A similar segregation of B cells/T cells to distinct regions occurs within the white pulp of the spleen and the MALT (likewise, under control of chemokines elaborated by SLO-resident cells).

Homing of Naive Lymphocytes to Secondary Lymphoid Organs

The body's complement of B and T cells consists of more than 10^9 cells, each of which may display a unique antigen receptor directed to a specific epitope. Thus, the frequency of B or T cells directed to a specific pathogen is extremely low (eg, a frequency of 1 of 10^4-10^6 naive B or T cells). The problem faced by the adaptive immune systems is that these rare, antigen-specific cells are randomly distributed throughout the body and must be rapidly mobilized to respond to invading organisms. These rare antigen-specific cells must first contact antigen, then activate, and generate effector cells capable of eliminating the pathogen. The solution to this problem is achieved through the process of lymphocyte recirculation through the SLO.

Lymphocyte recirculation of naive T cells and naive follicular B cells is controlled by the expression of distinct homing receptors (adhesion molecules) whose ligands are expressed on cells within the SLO. Naive $CD4^+$ and $CD8^+$ T cells within the bloodstream express on their surface the adhesion/homing receptor, CD62L (L-selectin). T cells entering the blood vessels encounter the CD62L ligand, a tetrasaccharide called Sialyl Lewis x, that decorates the mucinlike CD34 and GlyCam-1 proteins on the endothelium of specialized high endothelial venules (HEV) within the LN cortex. The interaction of CD62L (on the T or B cell) with its endothelial cell ligands induces the rolling of the lymphocyte. CCR7, a chemokine receptor (found at higher levels on naive T cells but present on naive B cells as well) is then triggered by the chemokines CCL21 and CCL19, which results in the activation of the $\alpha_L\beta_2$ integrin (LFA-1) and the arrest of lymphocyte migration. This arrest results in transmigration (diapedesis) of the lymphocyte across the HEV into the T-cell zone of the cortex. A little CCL19 and CCL21 is produced by the HEV cells, but these chemokines are mainly derived from fibroblastic reticular cells (FRCs) in the T-cell zone of the lymph node and then transcytosed by and displayed on the surface of endothelial cells of the HEV. The interaction between the CCR7 receptor on the arrested T cells and its chemokine ligand triggers the migration of the naive T cells through the HEV and into the LN cortex (parafollicular T-cell region). Naive B cells express CD62L and sufficient CCR7 to be first drawn in across HEVs into the T-cell zone, but because they express high levels of CXCR5, they are rapidly drawn into lymphoid follicles along a gradient of the CXCL13 chemokine made by stromal cells in the follicle (B-cell zone) including cells of nonhematopoietic origin called follicular DCs. If naive lymphocytes do not encounter antigen, they traffic through the LN cortex to the medulla, then exit the LN though efferent lymphatic vessels, and ultimately return to the bloodstream where the process of recirculation though the SLOs begins again. Lymphocyte trafficking through the spleen and MALT is likewise regulated by the interplay of adhesion receptor/ligand interactions and chemokines constitutively expressed in these SLOs. In the case of MALT, a set of unique receptor/ligand interactions (restricted to these sites) guide the trafficking of adaptive immune cells to these specialized mucosal sites.

Afferent lymphatic vessels are localized in all body surfaces/tissues, and they drain into the subcapsular sinus of the LN. Virus infection of a body surface/tissue triggers the innate immune system—the initial defense against infection. Innate immune cells located peripherally encounter pathogen, take up virus/viral antigen, are activated, and then migrate through afferent lymphatics to the draining LN. In the draining LN, these migrating cells (along with resident DC in the LN cortex) serve as APC to the naive B and T lymphocytes trafficking through the LN. As a result of encounter with antigen displayed on the APC, the lymphocytes with antigen receptors specific for the antigen or pathogen will no longer traffic through the LN, but rather will be retained in the LN and initiate the process of activation and differentiation leading to the generation of specific effector B and T cells directed to the pathogen. Thus, the SLO concentrate antigen in a specific site where the rare antigen-specific, naive B and T cells circulating through the body can encounter and respond to the pathogen. Once fully activated, effector T cells exit the lymph node, enter the blood, and instead of homing to SLOs, specifically migrate to the site of viral infection.

If infection by a virus is localized to the body surface (eg, papillomavirus infection of the skin or influenza virus infection of the respiratory tract), then the adaptive immune response to that pathogen will be primarily induced in the LN draining these sites. Virus infections that result in systemic spread (ie, viremia) will also induce adaptive immune responses in the spleen. Viruses that infect sites where MALT are prominent (eg, rotavirus infection in the Peyer patches of the small intestine) will trigger the local induction of the adaptive immune responses at the site of MALT localization.

Genes and Molecules That Mediate Adaptive Immunity

With genome sequencing of the human and other species (eg, the mouse), the overall organization and fine structure of most genetic loci that regulated adaptive immune responses are now well defined. Furthermore, the molecular structure of MHC molecules, T- and B-cell antigen receptors, and many other molecules essential for the adaptive immune response have been elucidated through analytical techniques (eg, x-ray crystallography). It is useful to briefly review the properties of certain genes and molecules—in particular, the MHC locus genes and proteins and the components of antigen receptors, costimulatory ligands, and receptors and coreceptors. Antibodies and the B-cell receptor will be discussed in a later section of the chapter. Cytokines and chemokines are frequently products of innate immune system cells but are also secreted by adaptive immune cells. Several of the cytokines that control adaptive immune responses will also be mentioned later in this chapter.

The Major Histocompatibility Complex Locus Genes/Products

The genes within the MHC locus are classified as class I (Ia and Ib), class II, and class III. The class I and class II proteins are intrinsic to the presentation of antigens to T cells. The MHC class I proteins are made up of a transmembrane α (heavy) chain and an associated β2-microglobulin (β2-M) protein. The MHC class II proteins are transmembrane αβ heterodimers. The class III genes encode proteins, some of which have important functions in immunity (eg, the complement proteins, C2, C4, and factor B [Bf]), but have no sequence structural homology to the MHC class I or II genes or products. Class III genes encoding cytokines of the TNF family (eg, TNF, lymphotoxin α and β [LT-α and LT-β]) also play a critical role in innate immunity. In the case of LT-α and LT-β (and related TNF family members), these gene products also play a role in the development of the SLO, because genetic deficiency in either LT-α or LT-β results in defective lymphoid tissue formation during development. The linkage of these and other class III genes to the MHC locus suggests a strong evolutionary pressure to retain these

genes in close proximity to the genes encoding the conventional MHC locus products. The importance of this linkage is evident considering that the class III genes encoding tapasin, the TAP1 and TAP2 transporter, and immunoproteasome subunits (b1i-LMP2, b5i-LMP7) all map within the MHC and regulate the induction of $CD8^+$ T-cell responses and the expression of $CD8^+$ T-cell effector activity. The other class III genes within the locus, however, encode proteins with no obvious immunologic function (eg, the gene for the steroid biosynthetic enzyme 12-hydroxylase, which when deficient causes adrenal hypoplasia). Unlike class Ia and class II genes, the numerous class III genes exhibit minimal polymorphism (ie, have few/no allelic forms), and the function of many of their products have yet to be defined.

The class Ib genes encode proteins whose overall structure is similar to the highly polymorphic class Ia gene products, but these genes display minimal polymorphism. The class Ib genes also encode cell surface molecules that are associate with β-2M. The number of class Ib genes varies among species, and the expression of these β-2M–associated class Ib gene products varies among class Ib products, both in magnitude and in tissue distribution. Many class Ib gene products serve as ligands for receptors on NK cells and/or γ:δ T cells.[45] The HLA-G molecule, for example, is expressed on fetal-derived placental cells along the uterine wall and provides an N-terminal leader sequence (signal peptide) that binds to the groove of HLA-E, which can then ligate an inhibitory receptor on maternal NK cells, CD94/NKG2A, to inhibit NK cell killing of fetal cells. These placental lining cells are also devoid of classic MHC class Ia molecules and are not recognized by $CD8^+$ αβ T cells; unlike other class Ia–deficient cells, these placental cells are not susceptible to killing by NK cells as a result of HLA-G (and HLA-E) expression. The class Ib HLA-E molecule is relevant for both NK cell activation and inhibition. HLA-E molecules bind a limited set of peptides derived from the signal peptides of class Ia molecules and HLA-G. These peptide/HLA-E complexes can not only bind to an inhibitory receptor on NK cells and prevent NK cell–mediated lysis, as mentioned above, but can also contribute separately in different contexts to NK cell activation through a distinct activating receptor. In contrast, the expression of products of the MHC class I chain–related (MIC) family of class Ib genes is controlled by a novel mechanism. These molecules, MIC-A and MIC-B, are selectively expressed on the surface of epithelial cells (and fibroblasts) undergoing stress (eg, heat shock and viral infection). The MIC-encoded molecules interact with receptors on NK cells, γδ T cells, and rare $CD8^+$ αβ T cells, activating these cells to kill MIC-expressing cells. At least one class Ib gene, HFE, has no apparent immune-related function. It encodes a protein expressed on the intestinal epithelium that is involved in iron metabolism. Deficiency in HFE gene expression results in the iron storage disease, hereditary hemochromatosis. As these examples suggest, the full spectrum of class Ib gene function is complex and not yet elucidated.

The hallmark of the class Ia genes, the genes encoding the classic transplantation antigens, is the extensive polymorphism of the HLA-A, B, and C locus genes. Approximately 3500 HLA-B locus alleles alone and a total of about 6000 HLA class I alleles at the A, B, and C loci have so far been identified. Because class Ia genes are codominantly expressed, individuals can express up to six different HLA class Ia gene products on their cells (ie, two alleles each of HLA-A, B, and C). X-ray crystallographic analysis of HLA class Ia molecules has demonstrated that most amino acid differences associated with individual class Ia alleles are localized to the outer two domains (α_1 and α_2) of the heavy chain molecule that encloses the peptide-binding groove and thus contribute to interaction with the TCR-αβ heterodimer on $CD8^+$ T cells.

Allelic variation in the α_1 and α_2 domains of the class Ia molecules is clustered within specific sites in the outer two domains; that is, in the floor of the peptide-binding cleft (consisting of a β-strand structure contributed by both the α_1 and α_2 domains) and along the two walls of the bivalve, clamshell-like peptide-binding cleft, demarcated by two α helical walls (ie, halves of the clamshell) contributed by the α_1 and α_2 domain, respectively, with polymorphic amino acid side chains pointing into the groove from the walls. The floor of this bivalve, clamshell cleft demarcates *pockets* defined by the polymorphic residues surrounding the pockets, which allow antigenic peptides with the appropriate amino acid side chains to be *anchored* in the groove. The location of these pockets in the groove (typically, two to three pockets in the floor of the class Ia–binding cleft), and the allele-specific residues of the α_1 and α_2 domain lining the pockets in the floor of the groove, defines the anchor residues of peptides capable of binding to the groove and, therefore, the MHC class I allele–specific, peptide-binding motif of antigenic peptides (and self-peptides) that bind to a specific class Ia allele. The interaction of other residues in the 8-10 amino acid antigenic peptide with the side chains of residues lining the wall of the groove further defines (limits) the range of peptides capable of stable interaction with a particular class Ia allele. Finally, once stably bound to the class Ia–binding cleft, the side chains of one or more amino acids of the bound peptide extend up and out of the surface of the groove and, therefore, are accessible to the TCR. X-ray analysis of crystals of TCR-αβ heterodimers and specific peptide/class I complexes indicates that the TCR interacts with a surface composed of amino acid along the α helical walls contributed by the class I α_1 and α_2 domains, and amino acid side chains of the bound peptide. Thus, both the class Ia molecule and the bound peptide contribute to the structure recognized by the TCR on $CD8^+$ T cells, either during T-cell development in the thymus (with self-peptides bound to the MHC) or in response to viral infection in the SLO or the site of infection.

Because each allelic class Ia molecule can only bind a limited range of peptides, dictated by the structure of the peptide-binding cleft, polypeptides encoded by a virus must contain one or more strings of amino acid in a precise linear arrangement to stably bind to a particular MHC class Ia allele and be recognized by $CD8^+$ T cells. Why then is the $CD8^+$ T-cell recognition of a foreign antigen by any individual limited to six possibilities (ie, the peptides captured by the two alleles of HLA-A, B, and C inherited from one's parents)? Why do we not express on our cells multiple versions of each HLA antigen to increase the chances of capturing an antigenic peptide and stimulating a $CD8^+$ T-cell response? The explanation for these questions likely comes from the process of $CD8^+$ T-cell selection in the thymus. The greater the number of MHC alleles expressed in the thymus (ie, displaying self-peptides), the greater the chance that the developing $CD8^+$ T cells will undergo negative selection (deletion in the thymus in response to TCR recognition of self-peptide/MHC complex), thereby decreasing the number of potential naive $CD8^+$ T cells capable of responding in the periphery to infection. The compromise is at the species level (ie, express a diverse array of MHC alleles within an outbred population). Consequently, although any particular individual displaying a particular set of MHC class Ia alleles may not be able to mount a response to a particular virus, sufficient allelic diversity exists within the population for most individuals to display an MHC allele capable of presenting at least one antigenic peptide to $CD8^+$ T cells and clear infection.

The MHC class II genes, HLA-$DP_{\alpha:\beta}$, $DQ_{\alpha:\beta}$, and $DR_{\alpha:\beta}$, also express a high degree of polymorphism (with the exception of the DR_α gene, which has a limited allelic polymorphism, for reasons that are not fully understood).[129] The six alleles of the codominantly expressed human MHC class II genes inherited by any individual may be further diversified by the ability of the α chain of the class II allele (eg, DR) to pair with the β chain of another class II gene product (eg, $DQ_{\beta1}$) forming a hybrid class II molecule with a new (additional) peptide-binding capacity. The polymorphism of class II genes, as with class Ia genes, is largely restricted to the gene segments encoding the domains forming the peptide-binding groove (ie, the α_1 and β_1 domains of the class II α and β chains, respectively). Unlike the ends of the peptide-binding groove of the class I molecule (which is *closed* and, therefore, capable of accommodating peptides of a restricted length—8-10 amino acids), the ends of the class II–binding cleft are open, allowing the class II cleft to accommodate peptides of variable lengths of 13 or more amino acids, containing N- and C-terminal amino acid extensions of the 8-9 amino acid internal residues representing the internal core of the antigenic peptides. As with the class I groove, the interaction of anchor residues of the peptides with pockets in the floor of the class II–binding groove is essential for the

selection of peptides with the proper binding motif by a particular class II allele. The amino acid residues in the N- and C-terminal extensions of the core peptide sequence, however, stick out of the ends of the clamshell groove and interact with regions of the α_1 and β_1 domains of the class II molecule outside of the binding groove to stabilize overall peptide/class II interaction. Therefore, the binding motifs of peptides that bind to particular MHC class II alleles is not as clearly defined and not as rigid as the motifs for peptides that bind MHC class I molecules.

The CD1 Family of Class Ib Genes and Lipid Recognition

The recognition of lipids as well as peptides is an important feature of the host response to infection (and possibly in tumor immunosurveillance and autoimmunity). One family of MHC class Ib–like molecules that are encoded by genes outside of the MHC locus is the CD1 family of glycoproteins. The CD1 family consists of five genes and proteins in the human (ie, CD1a-e). These molecules have the unique feature of binding lipids and presenting those bound lipids to T cells. As with most class Ib molecules, they associate with β-2M and are encoded by genes with limited polymorphisms, and display the domain structure (α_1, α_2, α_3 domains) typical of class I molecules. The binding cleft of the CD1 molecule, however, is deep and extremely hydrophobic (267). Thus, these molecules can efficiently bind and present lipids/glycolipids to T cells, with several exceptions; however, they do not bind peptides. These molecules are not retained in the ER after synthesis awaiting peptide loading (ie, do not require TAP transporter function for cell surface expression), but likely traffic to the cell surface, and then capture lipids, either at the cell surface or after recycling into endosomes. Therefore, lipid uptake by CD1 molecules is similar to the peptide capture mechanism employed by MHC class II molecules. Different CD1 family members bind distinctly different lipid moieties as dictated by the structural constraints of their hydrophobic binding groove.[17] They can bind both lipids of microbial origin (with mycobacterial lipids representing the best characterized moieties examined to date) and lipids/glycolipids of cellular (self) origin. The mechanism of lipid presentation by CD1 family involves the assembly in the ER of newly formed CD1 molecules with endogenous lipids, their transport to the cell surface, internalization into endocytic compartments where they can, with the help of saposins, exchange the endogenous lipid in the groove for exogenous lipids that may be pathogen-derived, or for other endogenous lipids derived from other cellular compartments. CD1 molecules are expressed on DCs, monocytes, thymocytes, and some B lymphocytes. Conventional TCR-αβ–displaying T cells can recognize certain glycolipids (eg, CD1d/mycobacterial mycolic acid complexes), and these lipid-specific T cells may play an important role in the host defense against infection with intracellular bacteria. Recognition of lipid-bound CD1 molecules appears, however, to be a major focus of NK T cells (T cells that do not express CD4 or CD8 coreceptors but do express characteristic NK cell surface markers and whose TCRs contain an invariant TCR-α chain).[80] The lipid/CD1 interaction, with the invariant TCR on NK T cells, induces the rapid production of cytokines (eg, IFN-γ, IL-4) by the NK T cells. The CD1 recognition system and the T cells responding to CD1 molecules appear to comprise an important evolutionary mechanism for responding to nonviral microbial infections. The contribution of the CD1 family and most other MHC class Ib molecules in the host response to virus infection may be limited.

T-Cell Receptor, Coreceptor, and Costimulatory Genes and Proteins

Specific antigen recognition by T cells is carried out by a heterodimeric disulfide-linked cell surface glycoprotein, the TCR. The TCR heterodimer is similar in overall structure to the F_{ab} portion of Ig molecules, reflecting that the TCR chains are members of the Ig gene superfamily. Thus, antigen recognition by conventional $CD4^+$ or $CD8^+$ αβ T cells is carried out by variable V_α and V_β domains of the TCR. Unlike the constant domains of antibody heavy chain genes that include many different constant regions genes that each give rise to a different antibody isotype or class, all αβ T cells utilize a single C_α gene segment and one of two virtually identical C_β gene segments downstream of two different sets of (duplicated) D and J gene segments. The same C region domains are utilized in the TCRs of both $CD8^+$ T cell and $CD4^+$ αβ T cells.

Coreceptors in the immune system bind the antigen receptor complex and assist in the initiation of antigen receptor signaling. They should be distinguished from costimulatory receptors, which do not participate directly in antigen receptor signaling. CD4 and CD8 are the two coreceptors, respectively, on helper and cytotoxic phenotype αβ T cells. CD4 is a single chain, glycosylated molecule containing four extracellular domains, the outermost of which binds MHC class II molecules. The CD8 molecule exists primarily as a disulfide-linked heterodimer glycoprotein consisting of an α and β chain, each containing a single extracellular Ig domain. Interaction with MHC class I is mediated primarily by the CD8α chain though the β chain of CD8 helps orient the heterodimer to appropriately bind MHC class I molecules. A subset of $CD8^+$ T cells express CD8 α/α homodimers and are localized primarily to the gut epithelium. These $CD8^+$ T cells may play a role in the recognition and destruction of stressed (eg, infected) epithelial cells. Both CD4 and CD8 molecules bind weakly to their respective MHC targets in the absence of TCR engagement. The cytoplasmic tails of both CD4 and CD8 molecules interact with a cytosolic tyrosine kinase (Lck) and, on TCR recognition of peptide/MHC complexes, delivers this kinase to the αβ TCR complex to initiate phosphorylation of the CD3 and ζ ITAMs (each CD3 chain tail has 1 ITAM, while each z chain tail has 3 ITAMs, adding up to a total of 10 ITAMs in the TCR complex). Phosphorylation of the two tyrosine residues in each ITAM leads to the recruitment of the ZAP70 tyrosine kinase, the subsequent phosphorylation of cytoplasmic and transmembrane adaptor molecules and the recruitment and activation of downstream enzymes leading to the induction of a range of activated transcription factors that contribute to the activation of genes relevant to T-cell proliferation and differentiation.

As mentioned above, activation of resting (primary) T cells requires both the activation of the αβ TCR complex (made up of the TCR, CD3 heterodimers, and the ζζ homodimer) as well as the separate engagement of the costimulatory receptor, CD28. The CD28 disulfide-linked homodimer (a member of the Ig supergene family) is constitutively expressed on resting T cells. CD28 interaction with its ligands, CD80/86, displayed on APC is required for the activation of resting $CD4^+$ T cell and $CD8^+$ T cells. Another member of the CD28 family, cytotoxic T lymphocyte antigen 4 (CTLA-4) (CD152), is expressed on activated T cells, but the interaction of CTLA-4 with the CD80/86 ligands delivers an inhibitory signal that limits the proliferation of activated T cells, thereby controlling T-cell expansion in response to antigen. Another CD28-related costimulatory molecule expressed on activated T cells is ICOS, which binds to its ligand displayed on APCs during T follicular helper cell generation.

Members of the TNF ligand superfamily, which are on activated T cells, can also participate in T-cell responses. The CD40 ligand (CD154) is expressed on activated $CD4^+$ T cells and on engagement of its receptor (CD40) on DCs and B cells, delivers a critical signal necessary for full activation and cytokine production by DCs, and for B-cell activation during responses to protein antigens. Other members of the TNF family expressed on activated T cells (eg, 4-1BB [CD137] and OX40 [CD134]) can also deliver costimulatory signals. In experimental influenza infection, blocking the interaction of OX40 with its ligand in the infected lungs results in efficient virus clearance but with a markedly diminished inflammatory response in the infected lungs.[198]

Integrins also play a critical role in T-cell activation and in the homing of naive and activated T cells to the SLO or sites of inflammation and/or

infection. The α_L β_2 integrin, LFA-1, is displayed on resting T cells, and through its interaction with its ligands, ICAM-1/ICAM-2, acts to enhance or stabilize the T-cell/APC interaction. LFA-1 expression is upregulated on activated T cells, and binding of LFA-1 to its ligands that are expressed at increased levels on inflamed vascular endothelium targets localizes activated T cells to inflammation sites. The α_4 β_1 integrin, VLA-1 (expressed very late in T-cell activation), also plays an important role in targeting effector T cells to infection sites through interaction with its receptor (VCAM-1) displayed on activated endothelium and is crucial for T-cell immune surveillance targeting viruses that can infect the central nervous system.

Antigen-Presenting Cells and the Induction of Adaptive Immune Responses

The hallmark of the adaptive immune system is specificity of foreign antigen recognition. For T cells, this means recognition of antigenic peptides bound to MHC class I or II molecules displayed on the cell surface. The T-cell response proceeds in two phases. First is the inductive phase: the activation of quiescent naive (or memory) T cells in response to recognition of specialized APC displaying the peptide/MHC complex, resulting in the generation of armed effector T cells. Second is the effector phase where cells displaying the appropriate peptide/MHC complexes are recognized by the activated T cells. The *classic* class I presentation pathway is ideally suited to generate the antigenic peptide/MHC class I complexes recognized by $CD8^+$ effectors in response to viral infection of any somatic cell type. Because antigenic peptide/MHC class I complexes are extremely stable, viral peptides displayed by MHC class I molecules displayed on the surface of infected cells are not transferred to surrounding uninfected cells. Consequently, the resulting destruction of bystander cells by $CD8^+$ T cells is negligible.

Due to the more stringent requirements for the activation of resting naive T cells, there has been considerable attention given over the past decade to the process of antigen processing/presentation by professional APCs (in particular dendritic cells or DCs) in the generation of peptide/MHC complexes during the inductive phase of the T-cell response. Currently, DCs fall into one of four main categories. The first category consists of conventional or classical DCs (both cDC1s and cDC2s); these are the most numerous DCs, and they arise from a bone marrow–derived, myeloid origin, common DC precursor. The second category, pDCs, are so-called because of their plasma cell–like morphology and surface expression of a CD45 isoform (ie, the B220 molecule, which, characteristically, is found only in B cells) and have been demonstrated to be the major source of IFN-α produced early (24-48 hours) after inflammatory stimuli, including virus infection. Thus, in addition to serving as a potential APC for T cells, pDCs serve as a major early innate immune defense against virus infection. They are derived from the bone marrow but from a distinct lymphoidlike precursor. The third category, monocyte-derived DCs, are a distinct subset of cells that assume a conventional DC-like phenotype but are induced during inflammation when monocytes differentiate into this DC lineage. Finally, Langerhans cells represent a distinct DC lineage that arises from the fetal liver and seeds the skin.

In support of their essential role in immune response induction, DCs (when mature) are 10-100 times more potent on a per cell basis as activators of naive T cells when compared with other immune cell APCs (eg, macrophages, B cells) and indeed are the primary APCs for naive T-cell activation in SLOs.

Given the critical role of DCs in the induction of the T-cell response (in particular, the antiviral $CD8^+$ T-cell response), viruses might be expected to have evolved strategies to inhibit the induction of $CD8^+$ T-cell responses in virus-infected DCs. As discussed elsewhere in this text, for individual virus genera, evidence indicates that infection of DCs by certain viruses may inhibit the APC capacity of DC.[167,170] Furthermore, productive virus infection of tissue DCs at body surfaces could also result in virus dissemination when the DC traffic to the SLO. These considerations, as well as the fact that certain viruses with narrow cellular tropism (eg., papillomavirus), therefore, are not capable of infecting DCs and providing polypeptides for processing via the classic class I pathway, led to the concept of cross-presentation of antigen (including, viral polypeptides) by DCs to $CD8^+$ T cells. Cross-presentation is largely limited to DCs, especially cDC1 cells, and is only relevant at the inductive phase of T-cell responses. This is the major mechanism for the induction of $CD8^+$ T-cell response to viruses such as papilloma and polio viruses, which presumably do not infect DCs (and for tumor cells, which cannot serve as professional APC). Most nonviral pathogens (eg, extracellular bacteria, fungi) trigger not only relevant $CD4^+$ T-cell responses but also some, presumably cross-presentation–induced, $CD8^+$ T-cell responses, even though the latter may not be relevant for the clearance of many of those pathogens.

Dendritic Cells Activate Naive T Lymphocytes in Secondary Lymphoid Organs

As with most microorganisms, viruses enter the body surfaces where the initial encounter with the immune system occurs. These body surfaces (eg, skin and mucosa of the GI, GU, and respiratory tracts) contain epithelial lining cells, which can serve as the initial cells supporting virus replication. The epithelial surfaces also contain DCs, which migrate to the SLO and deliver antigen to the adaptive immune cells that are trafficking from the blood to the SLO.

Dendritic cells localize to the epithelium within the epithelial cell layer (eg, Langerhans cells in the skin) at the junction of the epithelial basement membrane in GI and respiratory tracts, as well as in the mucosa and submucosa underlying the epithelial surface. The DCs at body surfaces are derived from circulating monocytic progenitors originating in the bone marrow, which are present in the blood and migrate to the body surfaces and differentiate into peripheral (tissue) DC. These tissue DCs exist as immature (inactive) cells at these sites, turn over at a low variable rate, and are replenished by the circulating blood monocyte progenitors entering the tissues.

Immature DCs are sessile cells that have a particular set of phenotypic markers or characteristics. They express low/intermediate levels of cell surface MHC class I and class II molecules; the latter (class II) molecules are found predominately not on the DC surface but within specialized endocytic vesicles. Immature DCs also express low levels of cell surface molecules such as CD80 and CD86, which serve as costimulatory ligands for naive lymphocytes, and also express other receptors of relevance such as CD40. These molecules play a critical accessory role in the triggering of naive antigen-specific (eg, virus-specific) T cells, ultimately leading to the generation of effector (ie, antiviral) T and B cells, and the formation of memory T and B cells directed to the pathogen. Immature DCs also have the ability to take up particulate and fluid-phase antigen into pinocytic/phagocytic vesicles with high efficiency. The enhanced capacity of these DC to take up material (including the remains of infected apoptotic cells) likely accounts for their unique capacity to serve as APC.

The initiation of an inflammatory response at the body surface (as produced by virus infection) triggers the DC activation (maturation) process. Activation of the tissue-resident DC results in the *de novo* expression/upregulation of chemokine receptors (notably, CCR7), which renders the activated DC capable of migrating out of the peripheral tissue, primarily through lymphatic vessels along a gradient of constitutively expressed CCR7 ligands, the CCL19/CCL21 chemokines produced by lymphatic endothelial cells. Activation of the tissue DCs is also accompanied by upregulated expression of MHC class I/II molecules and costimulatory ligands resulting in the maturation of the DC. Mature DCs

characteristically express high cell surface levels of MHC class I/II molecules and the costimulatory ligands, CD80/86 and CD40, which can be later triggered to induce or sustain DC maturation by activated $CD4^+$ T cells. Mature DCs migrate to the SLO where they act as the principal APCs for the induction of the adaptive immune response. Thus, DCs serve the dual role of delivery (concentration) of antigen to the SLO and acting as APCs to activate the rare antigen-specific T cells entering the SLO from the circulation.

Immature DCs can be activated by virus infection through at least three mechanisms:

1. Direct infection of immature DCs. The stimulus for DC activation/maturation is provided by the interaction of viral proteins or, more likely, viral nucleic acids with (and signaling through) microbial pattern-recognizing Toll-like receptors (TLR) expressed by DCs, and/or activation of intracellular antiviral signaling mechanisms mediated by the cytosolic RNA sensors including the retinoic acid–inducible protein I (RIG-I) and melanoma differentiation antigen-5 (MDA5) helicases, and RNA-activated protein kinase (PKR).

2. Uptake of virus and soluble antigen (or more likely apoptotic bodies enclosing portions of previously infected epithelial cells) by endocytosis or pinocytosis with activation of immature DCs through binding to one or more C-type lectin receptor(s) displayed on DCs with specificity for carbohydrates on viral glycoproteins/glycolipids or through the process of phagocytic uptake.

3. Activation/maturation of DCs (that have taken up viral antigens) by **inflammatory mediators** (ie, cytokines, such as TNF). These cytokines may be released by infected epithelial cells and tissue macrophages in response to TLR and/or intracellular viral signaling.

Direct infection of immature DCs and the subsequent migration of the activated/mature DC to the SLO would appear to be the most likely mechanism to ensure the induction of an effective antiviral adaptive immune response. Whereas this mechanism of viral antigen delivery to T and B lymphocytes in the SLO occurs, it begs the important question: *How do viruses with a very narrow cell tropism which are unable to either infect or express viral genes in hematopoietic cells like DC (eg, papillomaviruses) initiate an immune response?*

The capacity of immature DC to endocytose cellular debris/apoptotic remnants of infected epithelial cells provides an important mechanism for triggering adaptive immune responses. The endocytic uptake of virions or material from virus-infected cells may be the primary mechanism of viral antigen presentation to virus-specific naive $CD8^+$ T involving the *cross-presentation* of viral antigens. It is now recognized that of the two main categories of conventional DCs, called cDC1 and cDC2 cells, cDC1 cells are particularly competent in cross-presentation and activation of naive $CD8^+$ T cells, though like cDC2 cells they can also activate naive $CD4^+$ T cells. Antigen presentation and cross-presentation are described in more depth later in the chapter.

On entering the draining LN, activated (mature) DC migrates in response to a gradient of CCL19 and CCL21 to the paracortical T-cell regions in the vicinity of the HEV. Thus, antigen-bearing DC arrives at the precise location to interact with naive virus-specific T lymphocytes that have entered the draining LN from the circulation and, thereby, initiate the adaptive immune response.

Naive T lymphocytes have stringent requirements for activation that include both TCR antigen receptor engagement by the appropriate processed antigenic peptide/MHC complexes and engagement of costimulatory receptors (eg, CD28) displayed on naive T cells by their corresponding ligands (eg, CD80/86) along with peptide/MHC complexes by APC. Mature, antigen-bearing DCs are uniquely suited to carry out this process. The migration of these cells along with infectious virions (either cell-associated or free) from infected sites to the SLO initiate an immune response in the SLO and, more importantly, can spread infection to other sites in the body. One classic example of this is the dissemination of poxvirus from a peripheral infection site (body surface) to the SLO, where free (infectious) virions and infected cells carried from the inoculation site to the SLO can result in additional virus replication there, followed by virus entry into the bloodstream. Macrophages may serve as APCs in the induction of recall memory (secondary) immune responses at tissue sites where the requirement for memory T and B lymphocyte activation are less stringent. Indeed, mature migrant DCs that have entered the SLO in response to virus infection are often not the primary APCs for T-cell activation. LN-resident DCs can frequently capture viral antigen delivered by migrant DCs and present that antigen to naive T lymphocytes in the SLO—not by direct infection but by the transfer or "hand-off" of viral antigen from migrant antigen-bearing DCs to SLO-resident DCs. The relative contribution of direct antigen presentation by infected/antigen-bearing migrant tissue DCs, the cross-presentation of viral antigen by SLO DC, and the role of infectious virions delivered to the SLO to the induction of the T lymphocyte response have yet to be defined and will likely differ for different viruses. Intact virions and/or soluble (free, intact) viral antigen, however, undoubtedly contribute to the induction of effective antiviral antibody response by B lymphocytes.

Activation of T and B Lymphocytes in Secondary Lymphoid Organs

The precise localization of T cells, B cells, DC, and macrophages within the SLO is controlled by chemokines, which are largely produced by stromal cells of the SLO. In draining LN, naive (primary) T cells present in the circulation will enter the LN through the specialized HEV, where they encounter mature DCs displaying processed antigen bound to MHC molecules, which are localized to the HEV region (the T-cell zone). Most naive $CD8^+$ T cell and $CD4^+$ T cells entering the LN will not recognize the antigen (ie, they have TCR that fail to recognize the processed and presented antigen with sufficient avidity to activate the cells). These T cells will ignore the DCs and migrate from the LN cortex to the medulla, then enter the efferent lymphatics, and ultimately return to the bloodstream to continue their perpetual search for relevant antigen. A similar process of lymphocyte egress through the HEV occurs in the MALT. In the Peyer patches of the small intestine, antigen is taken uptake by mucosal DCs sometimes following direct viral antigen transfer through specialized intestinal epithelial cells, called microfold or M cells. Viruses that enter the bloodstream (causing viremia) as well as any antigen-bearing tissue DCs that, following maturation, gain access to the circulation will localize to the splenic white pulp. Here, circulating T and B lymphocytes enter the splenic white pulp by transiting through splenic arterioles where viral antigen-bearing DCs can encounter lymphocytes. The lymphocytes that do not recognize antigen will exit the splenic white pulp by entering trabecular veins and reenter the circulation.

The rare naive $CD8^+$ T cell and $CD4^+$ T cell that recognize processed antigenic peptides displayed on the APC surface in the SLO do so through TCR engagement. The activation of naive T cells leading to the induction of the adaptive immune response is a multistep process. For induction of antigen-specific (virus-specific) $CD8^+$ T-cell responses, the activation process requires interaction of the antigen-specific TCR chain complex with *processed* antigenic peptides presented by MHC class I molecules on the APC surface with sufficient avidity; TCR signaling is fully catalyzed by the interaction of the CD8 coreceptor on the T cell with a region of the MHC class I molecule distant from the site of peptide binding. The signals delivered by the TCR facilitated by the coreceptor are referred as signal 1. To fully activate naive $CD8^+$ (or $CD4^+$ T cells), activation of the CD28 costimulatory receptor on naive T lymphocytes by its ligands, CD80/86, which are displayed at high levels on mature (activated), antigen-bearing DC is required. Costimulatory signaling through CD28 constitutes signal 2, and the combination of signal 1 and signal 2 is required for

the activation and proliferation of naive T cells (Fig. 10.2).

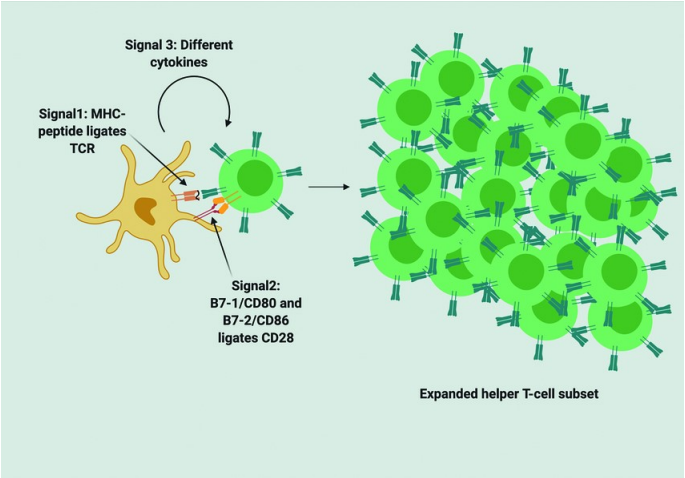

FIGURE 10.2 **T cells require two signals to be activated**. In the figure, the activation of naive $CD4^+$ T cells is depicted, but very similar processes activate naive $CD8^+$ T cells as well. Engagement of the T-cell receptor (TCR) on a naive $CD4^+$ T cell by MHC class II-peptide complexes on a dendritic cell induces TCR signaling (signal 1). Contemporaneous ligation of the CD28 costimulatory receptor on the naive T cell by the costimulatory ligands B7-1 or B7-2 (CD80/86) provides signal 2, resulting in the proliferation and activation of the original T cell to generate a clone of expanded effector T cells. Activation of T cells by cytokines is sometimes called signal 3.

The sequence of events resulting in the activation of antigen-specific naive $CD4^+$ T cell parallels the steps in $CD8^+$ T-cell activation, except that the antigen-recognizing TCR complex must bind (with sufficient avidity) the antigenic peptides bound to MHC class II molecules displayed by APC, and the CD4 coreceptor on $CD4^+$ T cells, in turn, interacts with a distinct site (outside of the region of peptide binding) on the MHC class II molecule. One important early consequence of $CD4^+$ T-cell activation by antigen is the upregulation of the expression of the CD154 molecule on activated $CD4^+$ T cells. CD154 (also called CD40 ligand or CD40L) is a member of the TNF gene family and, as its name implies, is a ligand for CD40 (a TNF receptor gene family member). CD40 is expressed on both activated/mature DC and B cells. Engagement of CD40 on DC and B cells by CD154 (CD40L) on activated $CD4^+$ T cells has important consequences for the function of these cells. Engagement of CD40 on DCs results in increased synthesis of certain cytokines (notably IL-12) by the antigen-bearing DC in the SLO, which promotes $CD4^+$ and $CD8^+$ T-cell differentiation into effectors. For B cells, the interaction between CD40-activated B cells and CD40L expressed on activated $CD4^+$ T cells is critical for the GC response and for B-cell differentiation into memory cells and antibody-secreting plasmablasts and plasma cells.

As a consequence of their interaction with antigen-displaying DCs, the naive T cells halt their transit through the SLO (ie, they are retained there). The APC/T-cell encounter leading to signal 1 transmission in the T cells appears to be of short duration (from 6 to 24 hours), after which the programming for T-cell activation/differentiation is independent of additional interaction with antigen. Delivery of signals 1 and 2, although necessary, is not sufficient in itself, however, to ensure full T-cell activation/differentiation and the generation of effector and memory T cells to the antigen. Additional signals, delivered by cytokines, can contribute to the T-cell activation/differentiation program in the SLO. These cytokines are primarily generated by cells of the innate immune system (eg, mature DC, NK cells, NK T cells), which enter the SLO in response to infection/antigen deposition in the SLO, and cytokine-derived signals that contribute to T-cell activation are sometimes called signal 3. IL-12 produced by mature (activated) DC in the SLO is a particularly critical cytokine for T-cell responses to viruses, because engagement of the IL-12 receptor on activated $CD8^+$ T cell and $CD4^+$ T cells promotes their differentiation into effector T cells with potent antiviral activity (eg, the generation of cytotoxic $CD8^+$ T cells).

Other cytokines produced during T-cell activation (eg, type 1 and type II interferons [INF-α or β and IFN-γ, respectively], IL-1, IL-4) by innate immune cells responding in the SLO to virus infection dramatically modulate the magnitude and type of both the effector and memory T-cell responses. These and other soluble mediators modify the course of T-cell activation/differentiation during the T cell's residence in the SLO.

Naive (primary) T cells are small resting cells, while activated proliferating T cells, called lymphoblasts, are larger. Proliferation of antigen-specific T-cell *clones* in the SLO leads to their differentiation into both effector T cells and memory $CD8^+$ T cell and $CD4^+$ T cells. T-cell proliferation is extremely rapid (cell cycle time of ≤6 hours) with estimates of individual naive T-cell precursors undergoing 8-20 divisions during this programmed proliferation. During their proliferative expansion and differentiation in the SLO, the responding T cells lose cell surface adhesion molecules (eg, CD62L) and receptors (eg, CCR7) necessary for naive T-cell circulation, and they upregulate the expression or express *de novo* adhesion molecules (eg, the β2 integrin, leukocyte function–associated antigen-1 [LFA-1] and β1 integrin, very late antigen-4 [VLA-4], respectively) and chemokine receptors (eg, CCR5, CXCR3) essential for effector T cells to localize to sites of inflammation. At the end of the proliferation/differentiation sequence, most activated antigen-specific effector T cells will exit the SLO (through the efferent lymphatics in the case of T cells responding in the draining LN), enter the circulation, and traffic to the site of infection/inflammation. The homing of activated T cells is controlled by chemokines released by innate immune cells (as well as by epithelial and endothelial cells and fibroblasts) at the site of inflammation/infection (eg, CXCL9 [Mig], CXCL10 [IP-10], CXCL11 [I-TAC] for CXCR3 and CCL3 [macrophage inflammatory protein-1α; MIP-1α], CCL4 [MIP-1β] for CCR5). This homing is also controlled by the inflammation-induced upregulation of integrin ligands (eg, intercellular adhesion molecule-1 [ICAM-1] for LFA-1 and vascular adhesion molecule [VCAM] for VLA-4) and adhesion receptors (P-selectin, E-selectin) on endothelial/epithelial cells at these sites.

The generation of effector T cells in the SLO responding to infection can occur in as few as 5-7 days in both experimental models and human viral infection. Given the rapid replication of most viruses, it is obviously essential to get specific T cells activated and mobilized as soon as possible through the concentration of antigen and the accumulation of the relevant rare antigen-specific T cells in the SLO, followed by rapid proliferative expansion/differentiation of these cells into effectors in the SLO. The rapid induction of an adaptive immune response to virus infection is not a foregone conclusion, however. The process of T- and B-cell activation can be delayed for weeks to months in the case of

infection with viruses such as HIV and HCV, which induce chronic infection.[77,131] In these instances, the virus has the capacity to suppress/delay the induction of the adaptive immune response.

The process of naive B-cell activation in the SLO is similar to naive T-cell activation. In the case of B-cell responses to viruses and other T-cell–dependent antigens, however, antigen-specific $CD4^+$ T cells play a critical role in driving B-cell activation/differentiation. The B cells that display a BCR antigen receptor (ie, the cell surface membrane-bound form of the Ig molecule that recognizes antigen) for an antigen present in the LN would encounter this antigen immediately after migrating out of the HEV in the paracortical (T-cell–rich area) of the LN. In the case of viral antigen, the relative contribution of free virus, soluble viral proteins, and viral proteins transported by migrant DC/macrophages from the infection site to the reservoir of antigen present in the SLO and available to specific B cells is not certain. Binding of antigen to the BCR (ie, cross-linking cell surface Ig) provides the initial stimulus (signal 1) for specific B-cell activation. Signal 1 delivery halts the constitutive migration of the B cell from the paracortical T-cell zone to the LN follicle and activates the chemokine-dependent migration of the B cells to the junction between the T-cell zone and the follicle. The strength of signal 1 can be greatly enhanced if the BCR coreceptor complex is ligated simultaneously with the BCR by interaction with complement components bound to antigen or antigen-antibody complexes. Cross-linking of the Ig receptor also induces internalization of the antigen-Ig complex into an endosomal compartment where the antigen is fragmented and associates with MHC class II molecules constitutively expressed by B cells. The antigen/MHC class II complexes are then cycled to the B-cell surface where they are available for recognition by antigen-specific $CD4^+$ T cells. Another consequence of signal 1 delivery (by antigen to B cells) is CD40 upregulation. The $CD4^+$ T cells, which interact with antigen-stimulated B cells, are themselves activated by prior encounter with antigen/MHC class II complexes on APC (ie, mature, antigen-bearing DC), and have upregulated CD154 (CD40L) expression. The activated antigen-specific $CD4^+$ T cells recognize the antigen/MHC complexes on the antigen-stimulated B cells and deliver signal 2 to the B cells in two forms: interaction of CD40L (on T cells) with CD40 (on B cells) and the release of T-cell–derived cytokines (notably, IL-4 and/or IFN-γ) whose synthesis is triggered in the $CD4^+$ T-cell TCR recognition of antigenic peptides displayed by the B cells. The fully activated (signals 1 + 2) B-cell lymphoblasts undergo programmed proliferation after migration to the follicles to produce GC, follicular structures consisting primarily of responding/proliferating B-cell lymphoblasts. The proliferation/differentiation of the GC B-cell lymphoblasts results in the formation of antibody-secreting plasma cells, which migrate from the LN to the sites of infection/inflammation, and more importantly to the bone marrow where the plasma cells serve as a depot of long-lived antibody-secreting cells. B cells that encounter antigen without $CD4^+$ T-cell *help* (ie, receive signal 1 but not signal 2) are capable of producing specific secreted antibodies; however, these antibodies will be of the IgM isotype and of low affinity. The isotype switch of antibody production from IgM to the IgG, IgA, and IgE isotypes, as well as the process of antibody affinity maturation, requires T-cell help (signal 2).

Viral Antigen Recognition by B Cells

Viral antigens are found on virions, on virally infected cells, as soluble molecules produced by virally infected cells, and as breakdown products from virions and infected cells. These antigens can be recognized by soluble circulating antibodies in plasma or tissues and by membrane-bound antibodies on the surface of B cells (ie, BCR).[2,51,74,134,147,152,157,179] If an individual has had previous exposure, particularly recently, to viral antigen(s) through infection or vaccination, then specific soluble high-affinity antibodies will likely be present in the plasma. A minority of these antibodies will be reactive with viral antigens presented on virions and virally infected cells and able to function antivirally. Most will be directed to a range of other viral antigens; they will be functionally inert but may be useful in the diagnosis of viral infection. In the case of prior infection or vaccination, memory B cells expressing specific antibody will be activated on contact with viral antigen, leading to a number of possible outcomes. One is the stimulation of these cells to enter GCs to undergo further rounds of affinity maturation as below. Another outcome is to differentiate to short-lived plasmablasts that will secrete soluble, specific high-affinity antibody and raise specific serum antibody levels, albeit on a limited time-scale. A third outcome is that the memory B cells, or subsequent GC B cells, will differentiate to form long-lived plasma cells in the bone marrow that will secrete specific antibody over long time periods, even decades. In the absence of a previous encounter with viral antigen, antibodies on the surface of naive B cells will bind antigen with relatively low affinity, setting in motion antibody affinity maturation and class-switching processes, which are discussed below.

Nature of B-Cell Epitopes

Most that is known about how antibodies recognize antigens at the molecular level has been determined from studies on the interaction of soluble antibodies or antibody fragments with antigen.[49,101,140,185,206] Given that B-cell surface antibody is essentially identical to soluble monomeric antibody, with an extra segment ensuring membrane localization, the conclusions should largely apply equally to cell surface antibody. Antibodies recognize molecular shapes, termed *epitopes*, on the surface of antigens. The antibody-combining site makes multiple contacts with the surface of the antigen to form complementary surfaces. The more complementary these surfaces are to one another—in terms of geometry and chemical character—the more favorable interactions will be formed between the antibody and antigen and the higher will be the affinity of the antibody for antigen. The affinity of the antibody for the antigen is one of the most important factors in determining the efficacy of the antibody *in vivo*.

The antibody-combining site is made up of residues contributed primarily from six highly variable segments or loops referred to as *hypervariable loops* or *complementary determining regions* (CDRs). The combining site can vary greatly in shape and character depending on the length and characteristics of the CDRs. Generally, most or all of the CDRs contribute to antigen binding, but their relative contributions vary. The heavy chain CDRs (particularly the third heavy chain CDR [CDRH3]) tend to contribute disproportionately to antigen binding. The CDRH3 in human antibodies can be very long, with a fingerlike appearance that could be used to bind into cavities on viral surfaces. The combining site of antibodies against smaller molecules such as carbohydrates and organic groups (haptens) are often more obviously grooves or pockets rather than the extended surfaces typically found in antiprotein antibodies.

On the antigen, epitopes come in as many different shapes and sizes as do antibody-combining sites. The area of antigen that contacts antibody, referred to as a *footprint*, is typically between about 400 and 1000 $Å^2$. All antibodies recognize a topographic surface of a protein antigen. Most usually, key residues in the epitope will arise from widely different positions in the linear amino acid sequence of the protein because of the manner in which proteins are folded. The linear sequence typically traverses from one side of the protein to the other a number of times. Such epitopes are described as discontinuous. Occasionally, key residues arise from a linear amino acid sequence. In such cases, the antibody may bind with relatively high affinity to a peptide incorporating the appropriate linear sequence from the antigen. Furthermore, the peptide may inhibit the antigen binding to the antibody. The epitope in such cases is described as *continuous*. An example of a continuous epitope would be a loop on the surface of the protein for which an antibody recognized successive residues in the loop. It should be noted, however, that an antibody

that recognizes a continuous epitope does not bind a random or disordered structure. Rather, it recognizes a defined structure that is found in the complete protein but can readily be adopted by the shorter peptide.

Antigenicity and Immunogenicity

A clear distinction is seen between the ability of an epitope to be recognized by antibody and its ability to stimulate an antibody response when presented to a host antibody system. A number of factors appear to be involved in how well a given epitope elicits an antibody response. One of the most important is the accessibility of the epitope on the protein surface.[118] Loops that protrude from the surface of the folded protein tend to elicit particularly good antibody responses. Sites that do elicit strong antibody responses on viral surface proteins will tend to be those in which mutations occur as the virus comes under selection pressure from the response in the phenomenon of neutralization escape. Figure 10.3 shows the sites on the hemagglutinin (HA) protein from the surface of the avian influenza virus that have mutated to avoid neutralizing antibody responses, with those at the "top" of the molecule distant from the viral membrane and most accessible to antibody. Natural infection and vaccination strategies typically induce antibodies to the top of HA and mostly to variable regions and therefore the antibodies tend to be strain specific. Changes within these immunodominant variable regions allow neutralization escape and dictate repeated annual influenza vaccinations to provide robust protection across large populations. More conserved regions, particularly within the stem of HA, induce weaker immune responses, but if these could be presented in a more immunodominant format, they might induce broadly neutralizing antibodies and thereby provide more universal vaccines to replace current annual vaccines.

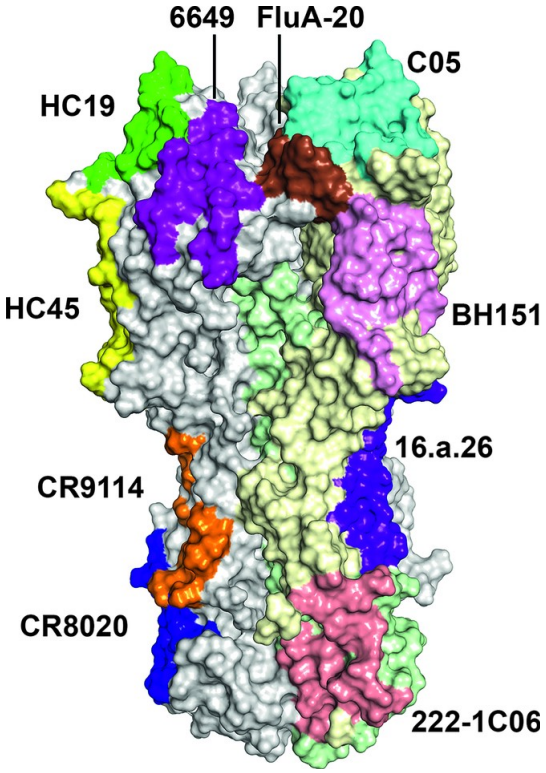

FIGURE 10.3 **Protective epitopes on influenza hemagglutinin (HA)**. The epitopes were determined from crystal and cryo-EM structures of Fab fragments derived from protective antibodies in complex with HA. The viral membrane is at the *bottom* of the figure. Many of the epitopes toward the top of the HA molecule are relatively variable between different influenza virus strains. FluA-20 targets a more conserved trimer interface epitope. The epitopes defined by broadly neutralizing antibodies CR9114, CR8020, and 16.a.26 in the stem of the HA molecule are also conserved especially in the different subtypes representing each of the two influenza A HA groups. These conserved epitopes are promising targets for more universal influenza vaccines. (Courtesy of Xueyong Zhu, Damian Ekiert, and Ian Wilson.)

Another factor that may influence immunogenicity is the precursor frequency of naive B cells bearing BCRs that can recognize features on the viral surface with reasonable affinity to trigger an antibody response.[3] Thus, it seems that the strong neutralizing antibody response elicited in many individuals by SARS-CoV-2 infection with the ancestral strain or vaccination with the S-protein from this strain is dependent, at least in part, on recognition of the ACE2-binding site on the receptor-binding domain (RBD) by naive human BCRs having a particular human heavy chain gene segment.[216] Such BCRs require little affinity maturation (see below) to bind the RBD with high-affinity and neutralize virus.

HIV is another virus in which highly exposed variable loops on the viral surface glycoprotein tend to be immunogenic.[210] Following primary infection, it takes some time (weeks) for neutralizing antibodies to reach a level where they interfere with virus replication.[160,201] These antibodies are often elicited to exposed variable loops on the virus. They are also elicited to regions of the HIV glycoprotein that, in certain isolates, lack glycans, so-called glycan holes in the glycan shield of the HIV envelope.[127] While antibodies are being elicited in early infection, the virus has diversified (ie, it has become a swarm of related viruses through the errors associated with reverse transcription of this RNA retrovirus). Among this swarm is a virus that has sequence changes in the epitopes targeted by the neutralizing antibody response that allows it to escape from the response. Extensive changes in loop length and sequence and "filling in" of glycan holes can occur with little or no reduction in the fitness of the virus so that the new virus becomes predominant. Eventually, a response is mounted to this virus and a second new virus emerges and so on. The antibody response chases the virus over many years but never appears to gain control.

An important consideration in relation to viral surface antigens and immunogenicity can be the spacing of the antigens on the virion surface.[11] It is well established that haptenated polymers, in which the haptens are spaced at 5-10 nm, can induce antibody responses in the absence of T-cell help. For vesicular stomatitis virus (VSV), in which the G protein is found in a highly organized quasicrystalline state on the virion surface, a strong immunodominant T-cell–independent neutralizing antibody response is induced by infection. A poorly organized recombinant form of VSV-G expressed in micelles induces a weaker B-cell response that is partially T-cell dependent. Soluble VSV-G alone, in the absence of adjuvant, fails to induce a B-cell response.

Generation of the Primary B-Cell Repertoire

The humoral immune system first senses the presence of novel viral antigens through the interaction of B-cell surface antibody (IgM and/or IgD) with antigen. Individual B cell express multiple BCRs of identical specificity. When these BCRs are occupied by antigen, the B cells are activated to divide and differentiate. The final results include the production of large amounts of specific, soluble high-affinity antibody and the generation of memory B cells that can be readily triggered to produce high-affinity antibody on repeated encounter with viral antigen.

The first encounter of viral antigen is with the primary B-cell repertoire of the individual. This repertoire is sufficiently large and diverse to recognize, with low or moderate affinity, in principle, essentially any molecular shape and, therefore, any viral variant. It is produced in B cells in the bone marrow by recombination of a limited set of germ-line segments. Hence, an individual's primary repertoire is unique to that individual and is in a constant state of flux. Briefly, the variable regions of antibody heavy and light chains are produced by V(D)J recombination in which numerous unique Ig genes can be made by joining different combinations of the V, D, and J segments at the heavy, and V and J segments at the light, chain loci. In humans, the potential heavy chain repertoire is ~45 V_H × 23 D_H × 6 J_H = 6.5 × 10^3 different combinations. Similarly, ~165 (33 V_λ × 5 J_λ) and 200 (40 Vκ × 5 Jκ) different combinations exist, for a total of 365 light chain (λ and κ) combinations. If we consider that each heavy chain could potentially pair with each light chain, then the diversity of the Ig repertoire is of the order of 10^6 possible combinations. Further diversity (junctional diversity) is generated because the joining of the V gene segments to (D)J is imprecise. In fact, the potential size of the human antibody repertoire is now estimated to be in the range of 10^{16}-10^{18}.[25]

Although mice and humans use combinatorial and junctional diversity as a mechanism to generate a diverse repertoire, in many species, including birds, cattle, swine, sheep, horses, and rabbits, part of the repertoire is generated by expression of a single functional gene with repertoire diversification being achieved by gene conversion, a process in which pseudo-V genes are used as templates to be copied into the assembled variable region exon. For example, in the chicken, during B-cell development in the bursa of Fabricius, rapidly proliferating B cells undergo gene conversion to diversify the antibody repertoire. Stretches of sequences from germ-line variable region pseudogenes, located upstream of the functional V genes, are introduced into the VL and VH regions. This process takes place in the ileal Peyer patches of cattle, swine, and horses, and in the appendix of rabbits.

B-Cell Activation by Viral Antigens

B cells can respond to both soluble and membrane-bound antigens, although it is likely that, *in vivo*, the most relevant interaction is with antigen on the surface of APCs.[13] The encounter of viral antigens with naive B cells expressing specific BCRs has two consequences that result in B-cell activation.[107]

First, a signal is transmitted into the B cell that is amplified via intracellular pathways and results in the activation of transcription factors that act to induce specific gene transcription promoting B-cell proliferation and differentiation. Cell signaling from the BCRs on naive B cells occurs when multivalent antigen clusters cell surface IgM (or IgD) molecules. The BCR is associated with a heterodimer of membrane-bound Igα and Igβ molecules, which bear immunoreceptor tyrosine activation motifs (ITAM) on their cytoplasmic domains. Following BCR clustering, tyrosines in the ITAM become phosphorylated to trigger a series of events that eventually lead to activation of transcription factors. Signaling by the BCR is modulated by coreceptors, most notably FcγRIIB and CD19. FcγRIIB is a potent inhibitor of B-cell signaling, and CD19 is an activating coreceptor. CD19 occurs linked to CD21, a receptor for the complement fragment, C3d. If, following complement activation, C3d has been covalently attached to viral antigen or to antibody-virus complex, the coreceptor complex can be linked to the BCR and BCR activation greatly enhanced.

Second, the BCR delivers viral antigens to intracellular sites where they are proteolytically cleaved, and some viral peptides are bound to MHC class II molecules. The peptide-MHC class II complexes are returned to the B-cell surface where they can be recognized by viral-specific TCR on $CD4^+$ T cells.[44] The specific subset of $CD4^+$ T cells specialized for helping B cells are T follicular helper (Tfh) cells, which can localize with B cells and provide help functions. The peptide-MHC class II complexes on the surface of the B cell stimulate the Tfh cell to produce cytokines and provide surface costimulatory molecules that promote B-cell proliferation and act on later generations of these B cells to promote differentiation to antibody-producing and memory B cells. The specific T helper cells have been primed earlier by DCs that present peptides to the T cells. Some viral antigens can activate B cells directly in the absence of T-cell help (so-called T-independent antigens).

It is important to note that the B-cell surface antibody and the TCR must recognize epitopes from the same molecular complex for the help signal to be delivered and B-cell activation to occur. Thus, a B cell that is expressing surface antibody to the surface protein of a virus could be activated by a T helper cell expressing a TCR to an internal viral protein if antigen challenge involved whole virions. A subunit vaccine consisting only of the envelope protein could not obtain help from such a T helper cell. Of interest, the process of shared T-cell help to B cells specific for a different viral protein may only occur for small viruses as the B cell and antibody responses to infection with vaccinia virus (a large pox virus used as the smallpox vaccine) are striking dependent on matched T-cell help.[171] This likely reflects a limit on the pathogen or particle size that a B cell can endocytose whole.

There are two pathways for B cells that have contacted specific T cells. In the first, after several rounds of proliferation, B cells can differentiate into plasmablasts in extrafollicular foci and secrete relatively large amounts of soluble antibody to the virion coat protein of our example. These antibodies are encoded by the unmutated germ-line repertoire and are likely to be of low to moderate affinity for viral antigen; they may be IgM isotype or class-switched to IgG or IgA.[47] The plasmablasts are short-lived with a lifespan of a few days. This early antibody is believed to be important as a first-line humoral response against viral infection. Some plasmablasts may migrate to the bone marrow and become long-lived plasma cells (see below).

In the second pathway established several days later and involving GCs in lymphoid tissues (see Section 2), the quality and versatility of the antibody to the virion coat protein can be greatly improved by affinity maturation.[3,44,47,59,87,93,105,109,194,196] In GCs[196] (see Section 2), B cells divide very rapidly, with cell cycles as short as 5-6 hours. Tfh cells are required for the formation and maintenance of GCs, they stimulate somatic hypermutation and selection of high-affinity clones, and they provide signals that regulate GC B-cell differentiation into plasma cells and memory B cells. Somatic hypermutation involves the introduction of nontemplated point mutations into V regions of the rapidly proliferating B cells at a rate on the order of 1 per 1000 base pairs per cell generation, which is ~10^6 times higher than the mutation rate of cellular housekeeping genes. The exact mechanisms by which mutations are introduced and preferentially targeted to appropriate V-regions, while constant regions of the Ig loci remain protected, are not clearly understood. Transcription through the target V region seems required, but is not necessarily sufficient, for somatic hypermutation. The enzyme activation-induced cytidine deaminase (AID) has been demonstrated to be essential for somatic hypermutation.[194] AID is a cytidine deaminase capable of carrying out targeted deamination of C to U and shows strong homology with the RNA-editing enzyme APOBEC-1. AID directly deaminates DNA to produce U:G mismatches. Selection from the vast array of B cells expressing slightly different mutated forms of cell surface antibody occurs ultimately on the basis of antigen affinity. B cells with the highest affinity for antigen acquire the most antigen, thereby leading to greater presentation of peptide/MHC class II complexes to T cells, which in turn provide the requisite cytokine signals for B-cell survival and selection. Although many B cells may seed a GC, only the descendants of a limited number of B cells will be present in the mature center.

Isotype switching, in which the constant region of IgM is replaced by that of another isotype leading to the expression of other isotypes, can frequently occur before the GC reaction as well as in GCs.[162] This will lead to the expression of, for example, IgG and IgA antibodies against the virion coat protein in the context of most viral infections. Isotype switching occurs through deletional DNA recombination events and involves repetitive DNA sequences or switch regions. The enzyme AID, described above, is critically involved in the process.

The B cells surviving the GC differentiate into plasma cells or memory B cells. Plasma cells accumulate in the spleen or draining LN during the early stages of acute infection, but as the immune responses subside, the majority of plasma cells are found in the bone marrow. These cells no longer express surface antibody but are committed to the secretion of soluble antibody—up to 10 000 antibody molecules per second. Because the half-life of IgG is of the order of 1-3 weeks, but specific antibody can be present for many years after antigen exposure, it appears that long-lived plasma cells maintain serum antibody levels and continue secreting antibody long after antigen has disappeared (see below). The half-lives of antibody responses in humans to measles and mumps viruses, for example, are estimated at more than 200 years.[8] Memory B cells can also last for many decades.[46,214] Memory B cells maintain surface antibody but secrete little if any antibody, unless activated. They divide slowly if at all. B-cell memory is considered in greater detail below.

Viral Antigen Recognition by T Cells

The T-cell response to viruses is largely mediated by T cells that utilize the TCR-αβ chain heterodimer to recognize a foreign antigen. One defining feature of the response of these cells is that TCRs recognize nonnative peptide fragments of foreign antigens (including viral polypeptides). Thus, in contrast to the immunoglobulin receptor (BCR), which is sensitive to antigen conformation, T-cell recognition of antigen is insensitive to three-dimensional antigen structure. Furthermore, T-cell recognition of antigens (eg, viruses) is primarily limited to the polypeptide constituents of the virus or, more precisely, to viral gene products expressed in infected cells, although some rare exceptions exist to this general rule, which are noted later in this chapter.

A second defining feature of the αβ TCR is that the antigen receptor recognizes these antigenic peptides bound to MHC molecules displayed on the surface of the antigen-containing (eg, virus-infected) cells. Thus, $CD4^+$ and $CD8^+$ T cells are restricted in their recognition of foreign antigen to cells displaying the foreign peptide complexed to the MHC molecule to which that T cell was selected during development in the thymus. This is the phenomenon of MHC restriction that defines antigen recognition by T cells. $CD8^+$ T cells will recognize cells displaying the foreign antigen peptide bound to one of several distinct MHC class I molecules expressed on the cell surface. $CD4^+$ T cells, likewise, are restricted in foreign peptide recognition by the cell surface MHC class II molecule to which the peptide fragment is bound.

The MHC locus is located on chromosome 6 in humans (chromosome 17 in the mouse) and extends over ~4 centimorgans of DNA (~4×10^6 base pairs). Two properties of the MHC locus product make it difficult for pathogens to evade the adaptive immune response. First, the MHC locus is polygenic. It encodes several different MHC class I and II gene products, and these differences in nucleotide/amino acid sequence control the range of peptide fragments that an individual MHC molecule can bind, so that every individual possesses a set of MHC molecules with a different range of peptide-binding specificity. Second, the MHC locus is highly polymorphic. Multiple versions (alleles) exist of each MHC gene product expressed in the population as a whole. In fact, the MHC genes are the most polymorphic genes in the human genome.

The MHC locus contains three major classes of genes: class I, II, and III. The MHC class I and II genes (and their products) were first identified because of their critical role as targets in graft rejection. Hence, the name for the human MHC locus (ie, HLA, for human leukocyte antigen locus), and H-2 or histocompatibility-2 locus for the corresponding genes in the mouse.

The MHC class I molecule is a heterodimer consisting of the MHC encoded 45-kd heavy (H) chain complexed noncovalently with a non-MHC locus–encoded small molecule, β-2 microglobulin (β-2M), which interacts with the H chain α_3 domain. Stable cell surface expression of the MHC class I molecule requires β-2M association as well as the presence of a peptide in the groove. Importantly, MHC class I molecules are expressed at varying levels on all nucleated cells of the body, with neurons being a notable exception. The expression levels of class I molecules on the cell surfaces can be upregulated by exposure of the cell to inflammatory mediators (eg, type I and II IFN). Therefore, the T cells that recognize peptide/MHC class I complexes (ie, the $CD8^+$ T cells) have the capacity to survey most body cells and interact with or destroy cells displaying the appropriate peptide/MHC class I complex. Three classical MHC class I loci (class Ia genes) exist in the human: HLA-A, HLA-B, and HLA-C (H-2K, H-2D, and H-2L in the mouse), and each gene is polymorphic (ie, displays multiple different alleles) within the human population.

Although both MHC class I and II molecules are similar in structure and function (ie, presenting foreign peptides), the difference in the cellular distribution of these molecules points to the role of the T-cell subsets ($CD8^+$ T cell and $CD4^+$ T cells) that recognize these molecules in the function of the adaptive immune system. For example, viruses have the potential to infect most nucleated body cells. Because these cells also express (to a varying degree) MHC class I molecules, they would be susceptible to recognition/destruction by activated effector T cells restricted by class I molecules in antigen recognition (ie, $CD8^+$ T cells). This simple association implies an important role of $CD8^+$ T cells in eliminating virus-infected cells and recovery from infection. By contrast, MHC class II molecules are primarily displayed on specific cells of the immune

system. Therefore, the main role of $CD4^+$ T cells, which recognize these molecules and the bound foreign peptides, is to activate and regulate the effector response of the other immune cells (eg, the recognition by activated $CD4^+$ T cells of peptide/MHC class II complexes on B cells to stimulate antibody production, the recognition of peptide/MHC class II complexes on macrophages harboring pathogens within intracellular vesicles to stimulate macrophage activation and pathogen destruction, and the recognition of peptide/MHC class II complexes on DC to stimulate cytokine production during the induction of adaptive immune responses).

Of course, the ability of $CD8^+$ T cell and $CD4^+$ T cells to interact with cells displaying foreign peptide/MHC complexes also depends on the ability of these foreign antigens to provide peptides to complex with the MHC class I and II molecules. As will be discussed below, the pathways by which foreign antigens gain access to and charge MHC class I and II molecules are distinct and dictate the contribution of $CD8^+$ T cell and $CD4^+$ T cells in the adaptive immune response to invading microorganisms.

Antigen Processing and Presentation to T Cells

The pathways of antigen processing and peptide presentation by MHC class I and II molecules to $CD8^+$ T cell and $CD4^+$ T cells were initially thought of as the *endogenous* and *exogenous* antigen presentation pathways, respectively, suggesting that endogenous proteins feed the class I pathway and peptides form exogenous proteins load MHC class II proteins; this initial notion is no longer helpful or even accurate. Indeed, from the viewpoint of viral immune responses, most naive $CD8^+$ T-cell activation likely involves exogenously derived viral proteins that are internalized by cDC1-type DCs and presented on MHC class I (often called cross-presentation). Similarly, endogenous cytosolic proteins are frequently captured in autophagosomes and reach the MHC class II pathway. The most useful way to segregate these two pathways is to recognize that for the MHC class I, pathway peptides are without exception generated in proteasomes, while for the MHC class II, pathway peptides are always generated in late endosomes/lysosomes. During viral infection, access to the cytosol and hence for proteasomal targeting is most efficiently achieved by infection of the cell by *de novo* synthesis of viral gene products. Infection of cells is the typical way that most virus-infected body cells are sensitized for recognition by activated $CD8^+$ T cells. Certain bacteria (eg, *Listeria monocytogenes*, *Salmonella*) and parasites (eg, malaria) can gain access to the infected cell cytosol, and these cytosolic pathogen-derived protein antigens can then access proteasomes, and the resultant peptides can enter the class I presentation pathway, as can strategies that introduce proteins directly into the cytosol of cells (eg, liposome-cell fusion). Certain cell types (most notably cDC1 type DCs) have the capacity to take up soluble or aggregated antigenic material (ie, apoptotic, infected cell debris) into phagosomes, allow these ingested proteins to leak into the cytosol, be processed in proteasomes, and thus have MHC class I molecules present antigenic peptides to naive $CD8^+$ T cells by a cross-presentation mechanism.

Protein antigens, both soluble and particulate (eg, bacteria, virions), which are internalized into the endocytic compartment of cells expressing MHC class II molecules, have the potential to enter the class II processing/presentation pathway and charge MHC class II molecules for recognition by $CD4^+$ T cells. Any foreign protein (including a viral membrane glycoprotein) expressed on an infected MHC class II–expressing cell can enter the class II pathway, if it can gain access to the endocytic compartment where processing occurs (Table 10.4).

TABLE 10.4 Features of viral antigen processing by the major histocompatibility complex (MHC) class I and class II presentation pathways

	Location	Proteolysis	MHC Loading Peptides
MHC class I pathway	Cytosol[a]	Proteasome[b] neutral pH	Endoplasmic reticulum
MHC class II pathway	Endosome[c]	Acid proteases low pH	Endosome[d]

[a]Viral proteins gain access to the cytosol by (1) *de novo* expression of viral proteins in infected cells; (2) direct virus-cell fusion at plasma membrane or in endosomes; (3) in specialized dendritic cells by phagocytic/endocytic uptake of virus or virus-infected cellular material into endosomes followed by endosome-ER fusion and retrograde transport of viral proteins in the cytosol.

[b]ERAP1 and ERAP2 aminopeptidases in the ER can modify the length of the viral peptide fragment produced by the proteasome after transport of the peptide into the ER by the transporter associated with antigen processing (TAP) transporter complex.

[c]Viral proteins in the form of virions and soluble and membrane-associated viral proteins must gain access to the endosome for fragmentation and binding to MHC class II molecules.

[d]Newly synthesized MHC class II molecules are blocked from loading peptides in the ER by the class II–associated invariant (Ii) complex, which is dissociated from class II molecules in the endosome by step-wise proteolytic cleavage of the Ii chain.

The steps in the processing (fragmentation) of polypeptides into peptides, as well as the transport and interaction of peptides with MHC molecules, not surprisingly, are different in the MHC class I and class II presentation pathways. Examination of these steps merits review because viruses have developed strategies to suppress the T-cell response by subverting the steps along these pathways.

Antigen Presentation by the Major Histocompatibility Complex Class I Pathway

Antigenic peptides bind newly synthesized MHC class I molecules in the ER of the antigen-bearing/infected cells. Therefore, the foreign polypeptide must be fragmented in the cytosol, peptides transported into the ER, and associate with a newly synthesized class I 45-kd heavy chain/β_2M chain complex before transport of the MHC/peptide complex from the ER through the secretory pathway to the cell surface (Fig. 10.4). Antigenic peptide production through fragmentation of cytosolic proteins is primarily carried out by the multisubunit, multicatalytic 20S/26S proteasome complex present in the cytosol. This enzyme complex accounts for most of the cytosolic protein degradation in all cells, and the proteasome is responsible for the generation of all self-peptides that are loaded on MHC class I during thymic selection. Two important

sources of proteasome substrates are newly synthesized self or foreign (eg, *de novo*) expressed gene products, such as viral proteins in an infected cell and preformed foreign microbes/microbial proteins that have gained access to the cell cytoplasm. The proteasome recognizes and degrades proteins ubiquitinated in a certain way, most commonly with at least four ubiquitin moieties attached to lysine 48 of the previous ubiquitin. This protein tagging involves the sequential action of the ubiquitin-activating enzyme (E1), several ubiquitin-conjugating enzymes (E2), and substrate-specific ubiquitin ligases (E3) to form polyubiquitinated proteins that can be captured by proteasomes. A significant fraction of newly synthesized proteins are misfolded and ubiquitinated. Defective ribosomal translation products may therefore be a major source of the protein substrates for MHC class I–binding peptides.

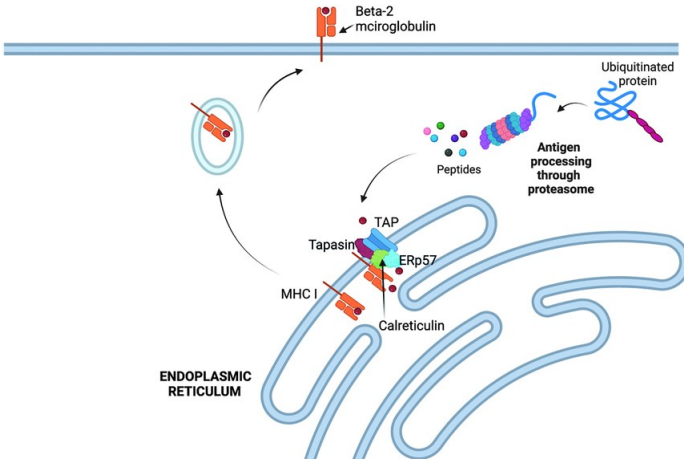

FIGURE 10.4 **The major histocompatibility (MHC) class I antigen presentation pathway**. Cytosolic proteins (or proteins that are delivered to the cytosol from outside the cell) are processively ubiquitinated. At least four tandemly attached ubiquitin units (in which the appropriate lysine residue of the ubiquitin, usually lysine 48, is modified by the next ubiquitin moiety) targets the protein to proteasomes. Peptide fragments are then transferred from the cytosol into the *ER* through the dimeric TAP (TAP-1/TAP-2) transporter complex. In the lumen of the ER, peptides with the appropriate anchor residues associate with the nascent 45-kd MHC class I/12-kd β2M molecules to form a stable complex, selection being assisted by the peptide loading complex that includes tapasin, ERp57, and calreticulin. Longer peptides are trimmed in the ER by the ERAP1 and 2 aminopeptidases, and vesicular transport results in transfer through the secretory pathway to the cell surface.

The proteasome is made up of a cap at either end and a core proteasome containing two identical outer α rings and two identical inner β rings, each ring containing seven proteins. The inner β rings contain three catalytic enzymes β1, β2, and β5 that preferentially cleave polypeptide substrates after hydrophobic and basic residues. This is noteworthy because MHC class I molecules preferentially bind peptides (in an MHC class I allele–dependent manner) with hydrophobic or basic *anchor* residues at their C-termini. Exposure of cells to certain cytokines (particularly, IFN-γ) induces the expression of three new proteasome subunits: iβ1, iβ2, and iβ5 (also called LMP2, MECL-7, and LMP7, respectively). These subunits replace existing subunits in the core proteasome, producing a second proteasome form, the immunoproteasome, that is present in IFN-treated cells. This replacement of the constitutive proteasome subunits with these inducible subunits changes the proteasome cleavage specificity, increasing the cleavage of polypeptides after hydrophobic residues and decreasing the cleavage after acidic residues (ie, peptides with acidic C-termini). Two of the IFN-γ–inducible proteasome subunits, iβ1 and iβ5, are encoded by genes within the MHC locus, suggesting a strong evolutionary selective pressure for the immunoproteasome in MHC function. IFN-γ also increases the production of antigenic peptides by inducing the expression of a multisubunit proteasome adaptor complex PA28 which, on binding to the ends of the core proteasome/immunoproteasome cylinder, increases the peptide efflux rate from the proteasome. Thus, a cytokine product, IFN-γ, produced by an innate immune cell type (NK cells) or adaptive immune effector cells (antigen-specific $CD4^+$ effector T cells) can enhance the display of antigen peptide fragments recognized by effector $CD8^+$ T cells. Another version of the proteasome, called the thymoproteasome, with a unique protease, iβt, is generated by cTECs, and this helps generate unique peptides for positive selection of $CD8^+$ single-positive T cells.

Proteasomes generate peptides 3-20 amino acids in length. Class I–bound peptides, in general, are 8-10 amino acids in length. Therefore, the proteasome peptide products that have the appropriate C-termini for MHC class I binding must also undergo cleavage at their N-termini. This is largely achieved in the lumen of the ER by the ER aminopeptidases ERAP1 and ERAP2. Overall, several studies have suggested that modifications of the proteasome core by inflammatory cytokines can facilitate the production of antigenic peptides (in cells infected with several different viruses), the recognition of which by $CD8^+$ T cells is dependent on these proteasome modifications and possibly the action of other cytosolic proteases.

Peptides generated in the cytosol are transported to the ER by the heterodimeric transporters associated with antigen presentation (TAP)-1/TAP-2 protein complex. This transporter complex is located within the ER membrane, forming a pore/channel there. Both proteins are members of the ABC family of adenosine triphosphate (ATP)-dependent small molecule transporters and are also encoded by genes mapping to the MHC locus. This transporter complex preferentially translocates peptide of 8-16 amino acids in length with hydrophobic or basic C-termini, and the TAP complex exhibits some restriction in transport efficiency of peptides with certain residues at the N-termini.

Newly synthesized MHC class I molecules must bind β-2M and have a stably bound peptide (of self or foreign origin) in their peptide-binding groove to gain the stable conformation necessary for it to exit the ER. This process is facilitated by a group of ER chaperones and enzymes that make up the peptide-loading complex. Within the ER, nascent class I α chain molecules are bound to the ER chaperone, calnexin until the 12-kd β-2M molecule binds, and calnexin is then replaced by the calreticulin scaffolding protein and the tapasin molecule (an MHC locus–encoded protein that forms a bridge between the class I α chain/β-2M complex and the TAP transporter). The binding of calreticulin and tapasin to the nascent MHC class I molecule stabilizes the complex, allowing the partially folded class I/β-2M complex to maintain a conformation (while it awaits a soluble peptide from the cytosol). The partially folded MHC class I molecule will selectively bind (select) only those peptides containing certain motifs; that is, common/conserved amino acid sequences distributed along the length of the peptide fragment. Each allelic form of the class I molecule has specificity for (binds to) a peptide containing an MHC class I allele, a specific peptide-binding motif. The ability of a specific MHC class I allelic variant to capture peptides displaying this conserved amino acid motif is dictated by the presence of polymorphic amino acid

residues in the binding cleft formed by the α_1 and α_2 domains. Another component of the peptide loading complex is the ERp57 thioreductase, which catalyzes disulfide interchange in the class I α chain during peptide loading. The ER luminal aminopeptidases ERAP1 and ERAP2 trims longer peptides. After proper peptide loading and folding, the stable class I/β-2M/peptide complex is freed from the chaperones and migrates from the ER to the cell surface. Note that, in uninfected cells, class I molecules still require a stably bound peptide to exit the ER and traffic to the cell surface. The sources of these *antigenic* peptides are degraded cellular (self) proteins.

The MHC class I presentation pathway is not restricted to proteins synthesized in the cytosol. Numerous examples are found of secreted and membrane proteins/glycoproteins that contain peptides that are recognized by $CD8^+$ T cells and, therefore, bound to MHC class I molecules. One way that these proteins can be degraded is if the polypeptide chains are generated in the cytosol as defective/mistranslated ribosomal products. Another way is retrograde transport of defective (misfolded) translation products that have been translocated out of the ER by the Sec61-dependent transport mechanism as part of constitutive ER-associated degradation or ERAD. Sec61 normally acts in the translocation of the translation products of membrane-bound ribosomes into the ER but can also shuttle intact (presumably defective) proteins in a retrograde fashion into the cytosol from the ER for degradation by the proteasome. The unique ability of professional APC (particularly cDC1 cells) to cross-present cellular constituents/fragments of virally infected cells may reflect the use of such a mechanism.

Antigen Presentation by the Major Histocompatibility Complex Class II Pathway

Antigen processing and presentation along the MHC class II pathway differs from class I presentation in several ways (Table 10.4). First, the class II pathway focuses primarily on antigens (both soluble and particulate) that typically enter cells by endocytosis or phagocytosis and are retained in endocytic compartments for subsequent processing (fragmentation) and loading of the peptide fragments into class II molecules. Cytosolic proteins that are captured by autophagy may also be delivered to endosomes and thus to the MHC class II pathway. The endocytic compartments of relevance to this pathway include early and late endosomes and lysosomes. Second, antigen fragmentation occurs in the low pH environment of the endosome (rather than in the neutral pH of the cytosol where proteasome-dependent MHC class I–binding peptides are generated). Third, both newly synthesized and recycled (from the cell surface) MHC class II molecules can capture and present peptide fragments to $CD4^+$ T cells. Fourth, unlike the strict length constraints (ie, 8-10 amino acids) for peptides bound to MHC class I molecules, peptides bound to class II molecules are at least 13 amino acids long and can be almost twice that length. As with the class I molecules, however, specific class II molecules (alleles) will selectively bind (select) peptides containing particular amino acids motifs (ie, peptides containing common/conserved amino acid distributed along the length of the peptide). As for class I molecules, these peptide-binding motifs are dictated by the presence of polymorphic amino acid residues in the class II-binding cleft (which differ for each class II locus gene product allele). In summary, class II molecules bind peptides produced from soluble/particulate antigen taken up by endocytosis in the same low pH compartment where protein fragmentation occurs. How then is class II processing achieved?

The MHC class II α and β chains, as with other membrane glycoproteins, are synthesized in the ER. After synthesis, the α:β chain heterodimer engages a type II membrane protein, the MHC class II–associated invariant (Ii) chain—invariant because the Ii gene is nonpolymorphic. The invariant chain forms a trimeric complex consisting of three class α:β heterodimers and three Ii subunits with each Ii subunit binding noncovalently to a class α:β heterodimer. This interaction of the class II α:β heterodimer with Ii serves two important functions. First, a region of Ii binds (with low affinity) to the peptide-binding groove/cleft of the nascent class II molecule in the ER and prevents the loading of peptides transported into the ER from binding to class II in this compartment. Thus, class II molecules do not bind the same spectrum of peptides as class I molecules, because they do not bind peptides in the ER. Second, Ii targets the class II molecules to the cell's low pH endosomal compartment (rather than the cell surface via the secretory pathway) where protein antigen fragmentation and peptide loading into the class II peptide–binding groove occurs.

Processing (fragmentation) of protein antigens in the MHC class II pathway is carried out by a variety of acid proteases, and at least one IFN-γ–induced lysosomal thiol reductase (GILT) located in the low pH endosomal compartment. Several related cathepsin cysteine proteases (eg, cathepsins S, L, B, F) and the unrelated cysteine protease, asparaginyl endopeptidase (AEP), have been implicated in antigen processing (fragmentation) in endosomes as inhibition of, or deficiency in, one or more of these proteases can affect the generation of certain antigenic epitopes recognized by $CD4^+$ T cells.

To bind peptides in the endosome, class II molecules must be liberated from Ii. This is achieved by the sequential cleavage of Ii by endosomal proteases (including, AEP, cathepsin S, and cathepsin L), freeing the class II α:β heterodimer from the membrane portion of the Ii, and leaving a short fragment of Ii (called *CLIP* for class II–associated invariant chain peptide), which remains bound to the peptide-binding groove of the class II molecule. CLIP must be removed from the class II molecule to make the class II–binding groove accessible to peptide fragmentation in the endosome, which is achieved through the action of a MHC-like molecule, DM (HLA-DM for human, H-2M for mouse), and which is encoded in the MHC locus and resides in the endosome. DM catalyzes the dissociation of CLIP from the class II α:β heterodimer. And, as with tapasin for class I molecules, DM stabilizes the class II molecule as the class II molecule interacts with, and sorts through, the myriad of antigenic peptides in the endosome, and it selects those peptides displaying the correct motif to allow these peptides to stably bind to the class II. It thus serves as part of the peptide loading complex that edits peptides for presentation by MHC class II molecules. This peptide selection process by class II molecules typically occurs in 2-4 hours (during the time that the class II molecules reside in the endosome). Once a stable peptide/class II complex forms, DM dissociates and the complex transits to the cell surface of MHC class II–expressing cells. *Empty* class II molecules (ie, lacking a peptide in the binding groove) are unstable. Consequently, in the absence of infection or specific exposure to foreign antigen (eg, vaccination), it is self-peptides derived from the endosomal proteolysis of cellular proteins (eg, soluble proteins taken up from the extracellular space by pinocytosis or the residues of phagocytosed apoptotic cells or cytosolic proteins captured by autophagy) that are bound to the MHC class II molecules on the surface of uninfected, class II–expressing cells.

Although *de novo* synthesized MHC class II molecules likely play a major role in capturing/presenting processed antigen to $CD4^+$ T cells, class II molecules can cycle back into the endosomes from the cell surface, and these *recycled* class II molecules may also capture/present antigenic peptide. Class II molecules containing self-peptides (or less frequently, class II molecules that escape the endosome and exit to the cell surface with CLIP peptide in their groove) appear able to reenter the endocytic compartment, exchange the bound peptide for a new peptide, and cycle back to the cell surface. By employing this recycling mechanism, MHC class II–expressing cells (in particular, professional APC, as with DC) would increase their chances of capturing and displaying foreign antigenic peptides to $CD4^+$ T cells.

Induction of T-Cell Responses to Antigen

The triggering of the T-cell proliferation program results in the rapid clonal expansion of antigen-specific T cells with about a 1000 times increase in specific T-cell numbers after 10-20 programmed divisions of individual specific precursors. This proliferative expansion of naive T cells is believed to occur primarily in the SLO. After antigen encounter, the proliferation/differentiation of T cells is rapid (ie, within 5-7 days) for most viral infections, leading to the generation of both effector T cells (and activated T cells that interact with [*help*] B-cell activation in the SLO) as well as memory T cells directed to the antigen (pathogen).

A major advance in the analysis of T-cell responses came with the development/application of MHC multimer technology, mainly involving MHC-peptide tetramers as probes for specific TCRs on flow cytometry. The development of this technology was based on two considerations. First, the realization that the formation of a stable MHC class I or II molecule required for the binding of peptide (with the appropriate MHC allele–specific binding motif) and, in the case of class I molecules, stable interaction with β-2M, allowed investigators to engineer soluble forms of the MHC class I molecules and express the molecules in a convenient expression system (eg, bacteria). The purified MHC molecule could then be refolded *in vitro* in the presence of a specific peptide corresponding to a known (or suspected) T-cell epitope to form a stable complex recognizable by the TCR on T cells directed to antigenic peptide/MHC complexes. Second, the antigen receptor on T cells (ie, the heterodimeric αβ TCR) does not undergo the affinity maturation exhibited by BCR and antibodies and, therefore, has a low intrinsic affinity for a single peptide/MHC complex (ie, estimated to average ~10^{-6} M), and the TCR presumably efficiently binds peptide/MHC complexes when the complexes are arrayed in a multivalent fashion on the cell surface. By forming multimers of refolded complexes of soluble MHC molecules and specific peptides, it was shown that stable interaction between peptide/MHC multimers and the TCR on the surface of the specific T cells could be achieved. By labeling the multimers with fluorescent tags, it was then possible to identify and quantitate antigen-specific T cells isolated from blood or tissue using flow cytometric methods. Another flow-based technique called *intracellular cytokine staining* (ICCS) relies on the ability of specific peptide—when complexed to the appropriate MHC allele to trigger the synthesis of cytokines *in vitro* by activated effector T cells whose TCR recognizes the peptide/MHC complex. By blocking cytokine release from the peptide-stimulated T cells (eg, with monensin/brefeldin A), the subset of T cells directed to the antigen can be enumerated by intracellular staining of accumulated cytokines with fluorochrome-tagged, anticytokine antibody. This technique has been extensively used to analyze the cytokine profile of T cells responding to acute or chronic viral infection (eg, HIV) in peripheral blood in humans and is routinely used to quantitate T-cell responses in both the SLO and peripheral sites in experimental virus infection models.

Effector Activities of B Cells

The antiviral activities of B cells are largely mediated by antibodies,[7,9,28,40,56,99,121,154,164] although B cells may have modulating effects on innate immunity. Antibodies are distinguished from T-cell effectors in that they have activity against free virus particles as well as against virally infected cells. They are the first line of defense against viral infection. In a very real sense, the adaptive antiviral activities of antibodies and T cells are complementary and should be considered together in attempting to understand immune responses to viruses. Nevertheless, it is revealing to consider the antiviral activities of antibodies at different layers of complexity[28]: *in vitro*, *in vivo* in the absence of other adaptive immune effectors as revealed by passive antibody transfer studies and *in vivo* in the presence of innate immunity and T-cell responses as in natural infection or following some vaccination strategies.

Antiviral Activities of Antibody *In Vitro*

As stated above, antibody can act against both free virus and infected cells. This is presented schematically in Figure 10.5 for an enveloped virus. Probably the most studied antiviral activity of antibody *in vitro* and one important for antibody protection *in vivo* is neutralization of free virus particles. One definition of neutralization is "the loss of infectivity which ensues when antibody molecule(s) bind to a virus particle, and usually occurs without the involvement of any other agency."[53] "Without the involvement of any other agency" (although rarely complement is included) indicates that neutralization is typically measured by simply incubating antibody, virus, and target cell together and demonstrating reduced infection. A second definition is "the reduction in viral infectivity by the binding of antibodies to the surface of viral particles (virions), thereby blocking a step in the viral replication cycle that precedes virally encoded transcription or synthesis."[97] Several mechanisms of neutralization of viruses likely operate. Perhaps one of the simplest mechanisms is based on steric obstruction of virus attachment or virus fusion and virus entry by the antibody molecule.[83,100,143,176,177] The relatively large bulk of the antibody molecule, very roughly similar to that of a typical viral spike for an enveloped virus, is suggested to be critical. Another mechanism of neutralization is inactivation of viral surface spikes for enveloped viruses. Many envelope spikes have relatively low stability—they are in conformations ("prefusion") such that interaction with host cell receptor proteins triggers extensive structural changes to permit the viral entry process.[133] Antibody binding can trigger conformational changes in such unstable spike proteins as shown in a number of instances, particularly for recombinant spike proteins,[133] and this is likely to be associated with neutralization. Aggregation of virions and prevention of viral egress (eg,[92,97,205,219]) from infected cells are other mechanisms described for neutralization, although the latter does not meet the strict definitions of neutralization above. Intracellular neutralization in which antibody intercepts virus in the cytoplasm of the target cell is a more recently described mechanism of viral inactivation that involves TRIM-21 degradation of nonenveloped viruses including adenoviruses, picornaviruses, and rotavirus.[19,62,200]

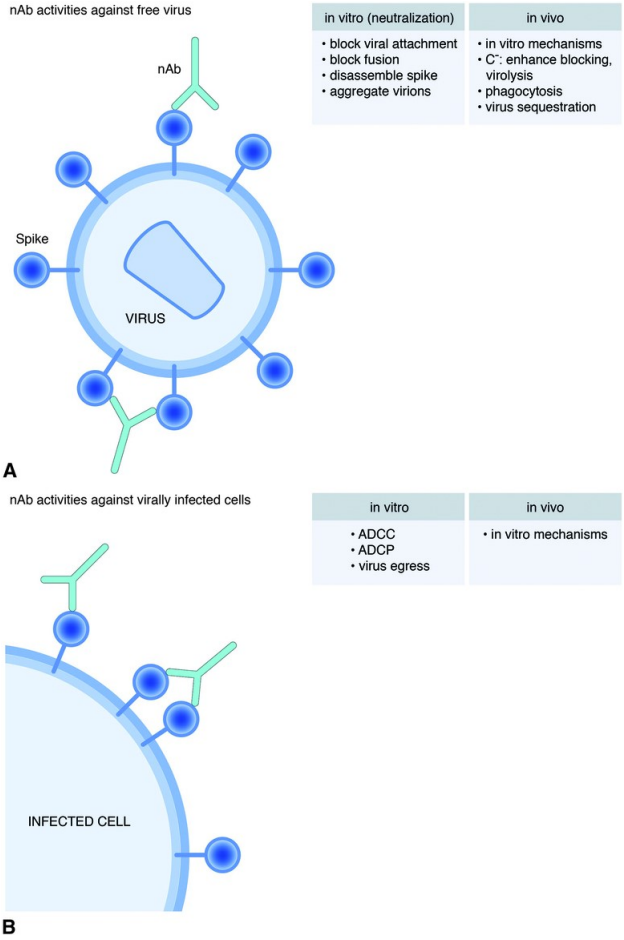

FIGURE 10.5 **Antibody activities against free virus particles and virally infected cells**. **A**. Activities against free virus particles. The primary activity is probably neutralization, whereby antibody molecules bind to virion surface proteins and block infection. Mechanisms of neutralization *in vitro* include blocking viral attachment to target cell receptors, blocking fusion of viral and target cell membranes, induction of conformational changes in virion surface protein that prevents entry, for example protein disassembly, and finally aggregation of virions. Virion-bound antibody molecules may also trigger lysis of virions via complement or phagocytosis of virions that can be assessed *in vitro* with complement or appropriate effector cells, respectively, and can be present *in vivo*. Sequestration by capture of virions on FcR-bearing cells and prevention of encounter with target cells is another possible *in vivo* mechanism of antiviral activity. An enveloped virus expressing only one surface protein is shown. **B**. Activities against virally infected cells. Neutralizing antibodies will bind to the proteins that are also expressed on virions (typically Env proteins). A range of activities can be assessed *in vitro* under appropriate conditions that may operate *in vivo*. Nonneutralizing antibodies will bind to proteins that are expressed on infected cells but not virions (eg, NS1 protein of dengue virus) or that are also expressed on virions but whose blockade may not lead to neutralization (eg, neuraminidase on influenza virions).

Mechanisms that invoke steric obstruction of viral entry by the relatively large size of the antibody molecule predict that the neutralizing efficacy of an antibody should to a first order be related to its affinity for antigen on the virion surface and the precise epitope recognized should be of lesser importance. Indeed, clear evidence exists that antibodies can neutralize viruses without binding directly to functional sites on the virion surface.[5,15,54,133,159,215] A particularly revealing instance of the importance of antibody size on a virion surface is the finding that antibodies to HA can interfere with the activity of neighboring neuraminidase molecules on the surface of influenza virus.[208] Models of neutralization based on steric interference by antibodies with viral entry can be very loosely termed *occupancy* or *coating* models, where the degree of coating of the virus surface by antibody to achieve neutralization differs between models. One model noted a striking linear relationship between the surface area of a set of viruses and the number of antibody molecules bound to virus at neutralization.[98,143] This implied that, for these viruses at least, a coating density corresponding to about half the available antibody sites occupied on the virus produced neutralization. This model is likely to be less applicable to complex viruses expressing multiple surface proteins (eg, the herpes or pox viruses).

One class of neutralizing antibodies that has received much attention in recent years is broadly neutralizing antibodies to highly antigenically variable viruses such as HIV, HCV, and influenza virus.[33,42] By definition, these antibodies recognize more conserved regions of the surfaces of highly variable viruses and have been much sought after as guides for the design of vaccines to the viruses.[28,29,75,104,156] Most recently, the emergence of variants of SARS-CoV-2 and the desire to have antibodies and vaccines to prevent future coronavirus pandemics has prompted searches for broadly neutralizing antibodies to coronaviruses, particularly to sarbecoviruses and more generally to beta-coronaviruses.[31,32,55]

Antibody activity against free virus particles can be augmented by Fc-mediated effector systems in several ways.[22,28,117] First, complement activation by virion-bound antibody and deposition of complement components on the virion surface can enhance neutralization.[180] Occupancy/coating models might argue that this reflects an increased coating of molecules on the virion that hinders productive interaction of virion and target cell. Second, complement activation can lead directly to virolysis by deposition of the terminal components of complement in the viral membrane. Third, Fc and complement receptors can bind antibody or complement-coated virions, leading to phagocytosis followed by inactivation in an intracellular compartment within the phagocyte. This process has been described *in vitro* for the picornavirus foot-and-mouth

disease virus (FMDV) and is believed to be important *in vivo* in protection against FMDV.[128] It is likely that neutralizing antibodies, which coat the viral surface, will be most effective in triggering virolysis or phagocytosis. An antigen present at relatively low density on the surface of a virion could, however in principle, bind antibody without mediating neutralization. Such nonneutralizing antibody could nevertheless trigger virolysis or phagocytosis.

Antibodies can act, not only against free virions but also against infected cells by binding to viral proteins expressed on the surface of virally infected cells (Fig. 10.5). Fc-mediated effector systems can lead to cell lysis or clearance via complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC). In the case of CDC, activation of the classic pathway by antibody complexing arrayed viral proteins on the infected cell surface can lead to C3b deposition and uptake by effector cells or the deposition of the terminal complement components in the infected cell membrane resulting in lysis. For ADCC, the antibody array on the infected cell surface is recognized by Fc receptors on effector cells triggering killing of the infected cells. Antibody can also inhibit (1) virus release or egress from infected cells (eg,[65,205]) by simply binding to viral proteins at the infected cell surface and (2) cell-to-cell transmission of virus[26,142,166] by mechanisms that are thought to be very similar to those that lead to virus neutralization. Higher concentrations of neutralizing antibody are generally required to inhibit cell-to-cell transmission than are required to inhibit infection by free virions,[4,84,114,142,158,166,186] although this not always the case.[186] Binding of antibody to the infected cell surface, particularly of neurons, has also been suggested to inhibit viral replication inside cells, presumably via signaling mechanisms.[63,113] It is expected that neutralizing antibodies will be effective against infected cells if they bind to surface molecule presentations on infected cells that are also expressed on virions. Antibodies that bind to molecules expressed on infected cells but not virions, for example, the NS1 proteins of dengue virus[78] or Zika virus,[204] can be effective against infected cells, although they are nonneutralizing.

Polymeric IgA and IgM have been shown capable of endosomal neutralization of virus. These antibodies are actively transported over the mucosal epithelium after binding to the polymeric Ig receptor and may, during transport, contact and neutralize transcytosing viruses.[18,36,64,70,94,124,125]

Finally, antibody can enhance viral infection *in vitro* under certain circumstances, generally in the presence of subneutralizing concentrations of neutralizing antibodies.[23] Enhancement is mediated by Fc receptors in some cases (eg, dengue virus)[71,73] but not in other cases where enhancement is observed with antibody Fab fragments.[183,184] The occupancy model of neutralization described above explains enhancement of infection as an effect that occurs at low occupancy of virion sites in the presence of permissive cells (eg, those bearing Fc receptors). As antibody concentrations increase, coating of the virus increases, eventually resulting in neutralization.

Antiviral Activities of Antibody *In Vivo*

The classic approach to determine the protective activities of antibody *in vivo* is to passively transfer immune sera or monoclonal antibodies to a naive animal, challenge with virus, and observe the outcome. This approach has consistently shown—for many different viruses, animal models, and challenge routes—a good correlation between the protection achieved and antibody serum neutralizing activity measured *in vitro*.[97,143] However, it should be noted that the correlation is not perfect, and there are many cases in the literature where there are varying degrees of breakdown of a linear correlation. For example, several broadly neutralizing antibodies, including some directed to the base of the HIV envelope spike (Membrane Proximal External region or MPER antibodies), appear to "punch above their weight" in that they are more effective *in vivo* than would be anticipated from their *in vitro* neutralization titers.[80,82,148] A similar deviation from a linear relationship between neutralization has been described for influenza antibodies[187] and a combination of SARS-CoV-2 neutralizing antibodies.[165] Antibody effector function triggered unequally by different neutralizing antibodies but yet important for protection may be contributory in these latter observations. It should also be noted that even a perfect correlation between neutralization and protection does not necessarily mean that neutralization is the decisive mechanism of protective activity. Neutralizing antibodies are probably those that bind most effectively to free virions and to virus-infected cells (at least for many enveloped viruses) so that, in principle at least, any of the mechanisms of antiviral activity presented in Figure 10.5 could be operative in protection.

Generally speaking, protection is achieved when neutralizing antibody titers in the serum of the animal at the time of virus challenge are relatively high, usually of the order of 1:100 or higher. In other words, the serum of the animal can be diluted 100-fold and 50%-90% (depending on the study) neutralization achieved *in vitro*.[28,148] This suggests that considerable apparent over-capacity with regard to neutralization is required to achieve protection. In some instances, protection can be described as sterilizing in that no evidence is seen of viral replication following challenge. Serum neutralizing titers (IC80) of 1:380 or greater provide sterilizing immunity in the lungs of cotton rats challenged with respiratory syncytial virus (RSV)[151] and titers of 1:700 (IC50) provide sterilizing immunity against challenge of macaques with chimeric simian-human immunodeficiency virus (SHIV) in an aggregate comparison of studies with different viruses, neutralizing antibodies and challenge routes.[148] In other instances, such as challenge with lymphocytic choriomeningitis virus (LCMV) in a mouse model[209] and with Ebola virus in a guinea pig model,[144] high titers of neutralizing antibody do not provide sterilizing immunity but do prevent disease. In most instances, however, it has not been established whether sterilizing immunity has been achieved.

A number of possible explanations exist for the apparent over-capacity in neutralization activity required for protection in many animal studies. If the assumption is that neutralization is the dominant protective mechanism, then for some viruses/animal models/challenge routes, it may be necessary for protection for antibody to "mop up" essentially every last virus particle requiring an excess of antibody. With the same assumption, the protective activity may be achieved at a tissue site with a lower antibody concentration than that of serum, again leading to an apparent over-capacity. Alternatively, protection may require an additional activity of neutralizing antibody distinct from neutralization. For instance, it may be that activity against infected cells, as well as (or in some cases even instead of) activity against free virions, is needed to provide protection. This would be consistent with observations that antibody concentrations required for, for example, blocking cell-to-cell transmission are typically higher than required for neutralization of free virus particles.

Animal model studies provided early evidence that mechanisms other than neutralization can be important in protection by neutralizing antibodies. In some cases, protection was found to be as effective with $F(ab')_2$ fragments, which lack the Fc domain and, therefore, are unable to trigger effector functions, as the corresponding whole IgG molecule. In other cases, however, $F(ab')_2$ fragments that are equally active as whole IgG molecules at neutralization *in vitro* were ineffective at protection. For yellow fever virus,[168] it was shown that neutralizing mouse IgG1 antibodies (poor activators of effector functions) are ineffective at protection, whereas IgG2a molecules (good activators) of the same specificity was effective. In many of the early examples in mouse models in which the Fc part of IgG is important, protection was independent of

complement.[143] This implied that protection by neutralizing antibodies in these cases most likely required activity against infected cells and involves ADCC or phagocytosis. More recently, antibodies engineered to reduce or eliminate or enhance complement activation and/or Fc receptor (FcR) binding have been used to study the contributions of effector functions to antibody protection and therapy. The overall picture that has emerged has been that the contribution of effector function, particularly FcR-mediated, to protection varies with the antibody-virus combination considered even for antibodies directed to the same viral target protein and that effector function tends to be more significant for therapy than for protection.[22,95,117] For example, FcR binding has been shown to contribute to protection against SHIV in monkeys for one bnAb but not for another more potent bnAb directed to a different site on the HIV Env protein.[72a,79,81,145] Effector function does contribute to clearance of virus in monkeys by nAbs, but the effects are modest and enhanced FcR binding does not enhance clearance and even decreases it.[10,199] In mouse models of HIV infection, FcR binding enhances virus control but only over certain nAb titer ranges.[24] For influenza, nAbs to the stem region are critically dependent on effector function for protective activity, whereas those to the head region are not.[52] For Ebola virus, protective antibodies show varying dependency on FcR interactions.[21] For SARS-CoV-2, nAbs have been described whose protective activity is influenced by effector function activity and those whose activity is independent of such activity.[165,207] Therapy of SARS-CoV-2 infection is more dependent on effector function than prophylaxis.[207]

Many examples of protective activity are exhibited by nonneutralizing antibodies in passive transfer studies in animal models. This activity appears to be directed at infected cells (Fig. 10.5) and generally appears to be somewhat less potent than that of neutralizing antibodies. For instance, a number of cases have been reported where neutralizing antibodies are protective against higher challenge doses or more pathogenic viruses than nonneutralizing antibodies. In many cases, protection by nonneutralizing antibodies is shown to depend critically on the Fc part of the antibody molecule and to occur in complement-deficient mice, suggesting that ADCC or phagocytosis may be crucial in clearing antibody-complexed infected cells. Protection with nonneutralizing antibodies is mostly restricted to those antibodies directed against enveloped viruses.

Passive transfer of polyclonal IgG preparations from immunized animals or convalescent humans has been shown to be effective in prophylaxis for several viruses, including hepatitis A virus, HBV, HCV, HSV, measles virus, rabies virus, RSV, smallpox virus, and varicella-zoster virus (VZV). Currently, three purified hyperimmune globulin preparations from human donors are in clinical use to prevent against serious disease due to HBV, human cytomegalovirus (HCMV), and VZV. A polyclonal antibody preparation against rabies virus, in combination with vaccination, is used as a postexposure prophylactic after virus exposure. The first monoclonal antibody, a humanized, neutralizing anti-RSV monoclonal antibody has now been in clinical use for more than two decades to protect at-risk infants.[68] However, increasingly a new generation of highly potent broadly strain cross-reactive nAbs ("super-antibodies") are being considered for clinical use.[197] These nAbs are typically generated by deeply mining the responses of convalescent individuals with potent neutralizing sera using single B cell isolation approaches. During the COVID-19 pandemic, many potent nAbs were isolated from convalescent donors and shown to have prophylactic effect in animal models and then in humans.[69] A large-scale clinical trial of an anti-HIV nAb showed complete protection against exposure to sensitive viruses with serum neutralizing titers of the order of 100s × *in vitro* neutralizing titer, concordant with the discussion above.[41] In the case of HIV, given the known properties of the virus, it is likely that the nAb, where protective, provided sterilizing immunity, that is, there was no significant replication of virus. In many cases involving passive nAbs, it is unlikely that the titers of transferred antibody achieve the levels necessary to offer sterilizing immunity, although this has not been widely studied. Rather, it would seem that neutralizing antibody sufficiently blunts the infection to allow the development of other protective mechanisms, presumably a $CD8^+$ T-cell response, active antibody responses, and innate immunity (see below). The protection of young infants by maternal neutralizing antibodies likely falls into this category. Maternal antibodies may "attenuate infection during the initial months of life, thereby creating optimal conditions for the natural immunization of the child as a result of infection."[220]

In the case of nAbs and established viral infection, the dogma has tended to be that they are relatively ineffective, particularly the later in the infection the antibodies are administered. For example, the RSV nAbs palivizumab and motavizumab are clinically effective prophylactically but not therapeutically.[86,155] SARS-CoV-2 nAbs have been shown to be effective prophylactically and when administered shortly after the onset of infection but not later in infection.[69] However, the dogma of lack of nAb efficacy in ongoing more mature infection is being increasingly challenged by the availability of highly potent cross-neutralizing Abs.[197] Thus, bnAbs are being investigated for a role in HIV therapy and cure,[12,38,136] and anti-Ebola nAbs are highly effective in monkeys in preventing death after symptom onset and appear also effective in symptomatic human infection.[43,90,146,153]

Many important human viral pathogens gain entry to the host via mucosal surfaces. Passive transfer studies show that antibodies present in the mucosal compartments at the time of exposure can protect against viral challenge.[50,94,137] Both mucosal secretory IgA (sIgA) and systemic IgG have been shown to be effective. Dramatically, nonneutralizing IgA can protect against rotavirus challenge in mice by an intracellular neutralization mechanism.[27]

Effector Activities of T Cells

The categorization of αβ TCR T cells into $CD8^+$ and $CD4^+$ subsets not only demarcates the MHC molecules that restrict recognition (ie, class I or class II) but also has functional significance at the effector level (Table 10.5). $CD8^+$ T-cell effector cells ($CD8^+$ T_E) are capable of destroying antigen-displaying cells (eg, virus-infected cells expressing the appropriate peptide/MHC class I complex) by direct cell-to-cell contact. $CD8^+$ T_E also produce/secrete several cytokines with antiviral activity (eg, IFN-γ, TNF-α) on TCR engagement. $CD4^+$ T-cell effectors ($CD4^+$ T_E) primarily function by release of cytokines (notably, IFN-γ, IL-17 and IL-22, and IL-4, IL-5, and IL-13) in response to antigenic stimulation. $CD4^+$ T_E, however in some instances, can also acquire killing capacity, that involves the same genes used by $CD8^+$ T cells, and they can kill by direct contact with cells displaying the appropriate peptide/MHC class II ligand recognized by the TCR complex. The differentiation of the naive T cells into T_E, in particular naive $CD4^+$ T-cell differentiation into T_H1, T_H2, T_H17, or T_{FH} cells, is regulated by a variety of factors (as discussed below).

TABLE 10.5 Effector activation of activated $CD8^+$ and $CD4^+$ T cells

Effector Type	Target Cell	Effector Mechanism
$CD8^+$ T cell	MHC class I^+	• Direct cytolysis (cell-cell contact) • Granule exocytosis (perforin/granzyme) • FasL (T cell)—Fas (infected target cell) interaction Cytokine synthesis • IFN-γ, TNF
$CD4^+$ T cell—T_H1	MHC class II^+	Cytokine synthesis • IFN-γ, TNF
$CD4^+$ T cell—T_H2	MHC class II^+	Cytokine synthesis • IL-4, IL-5, IL-13
$CD4^+$ T cell—T_H17	MHC class II	Cytokine synthesis • IL-17A, IL-17F, IL-22

MHC, major histocompatibility complex; IFN, interferon; TNF, tumor necrosis factor.

$CD8^+$ Effector Mechanisms

The $CD8^+$ effector T cells or CTLs destroy *target* cells (eg, virus-infected cells) by direct cell-to-cell contact, by two distinct mechanisms. One mechanism is by granule exocytosis.[79] Mature (fully differentiated) $CD8^+$ CTLs contain lytic granules within their cytoplasm, and TCR engagement on the CTL by peptide/MHC class I complexes on the target cell results in the formation of the immunologic synapse. In response to TCR engagement/synapse formation, these cytoplasmic granules are directed by the cellular microtubule-organizing center along actin filaments to the synapse formation site where the granule contents are released into the synaptic cleft. The adhesive interaction between the CTL and the target cell is stabilized/strengthened through the interaction of LFA-1 (on CTLs) with its ligand, ICAM-1 (on the target cell). The lytic granules contain a protein, perforin, which has a sequence homology to the complement component C9. The granules also contain a set of serine proteases, known as *granzymes* (for granule enzymes), which are present in a proenzyme form within the CTL granule and which are activated (by granule-associated cathepsin C) on granule exocytosis. Because of its homology to C9, some of the perforin monomer released onto the target cell can intercalate and polymerize (in the presence of extracellular Ca^{++}) forming a pore within the target cell plasma membrane allowing the granzymes access to the target cell cytosol. However, most of the perforin and granzymes initially stay bound together with serglycin and granulysin (the latter exists in humans but not in rodents) and are endocytosed by target cells at the immunological synapse formed between the $CD8^+$ CTLs and their targets. It is in acidified endosomes that most perforin activation occurs, endosomes are disrupted, and released granzymes are transferred into the cytosol. The granzyme family of serine proteases consists of at least 10 members in humans (including granzyme A, B, C, and K). They function by activating cell death pathways in target cells, including both direct activation of caspases and indirect activation of the mitochondrial pathway of caspase activation and apoptosis. The best studied granzyme, granzyme B, is present in both $CD8^+$ CTLs and NK cell lytic granules and acts by proteolytic cleavage and activation of procaspases (procaspase 3 → caspase 3) and proapoptotic Bcl2 family members (eg, BID → tBID) resulting in the DNA fragmentation and mitochondrial damage characteristic of apoptosis. An additional granule-associated lytic molecule, granulysin, can form pores, primarily in bacterial membranes, and may play a role in the destruction of certain bacteria by direct lysis.

CTLs can also destroy target cells by the upregulation (in response to TCR engagement) of the TNF family protein, CD154 (FasL, CD95L) on the CTL, and engagement of its receptor, the TNF receptor family member, CD95 (Fas), on the infected target cell. CD95 engagement results in the recruitment/activation of caspases and apoptotic death of target cells. Studies in experimental models of virus infection suggest that both the Fas/FasL and the granule exocytosis mechanisms can contribute to the elimination of virus-infected cells, and virus clearance by $CD8^+$ T_E.[192] The relative contribution of each lytic mechanism to virus clearance differs for different viruses.

The $CD8^+$ T_E also produce a number of cytokines in response to TCR engagement by antigen, most notably IFN-γ and TNF-α. The production/secretion of these effector cytokines appears to be tightly regulated and requires TCR-dependent signaling in the $CD8^+$ T_E. IFN-γ exhibits its antiviral effects through upregulation of MHC molecule expression and stimulation of the MHC class I processing/presentation pathway in infected cells. Because IFN-γ is a potent activator of tissue macrophages, activated macrophages would also have the capacity to take up and destroy virus/virus-infected cells. TNF-α, as can the other member of the TNF ligand family, CD154 (FasL), induce apoptosis of infected cells expressing the receptor for TFN-α and also can signal the production of proinflammatory/antiviral cytokines through receptor-dependent NF-κB activation.

$CD4^+$ T_E Effector Mechanisms

An essential role of $CD4^+$ T_E in antiviral immunity is to provide *help* for responding B cells in the SLO in the production of antiviral antibody. $CD4^+$ T cells also play a role, not yet completely defined, in the development and maintenance of the memory $CD8^+$ T-cells response during virus infection.[173,203] $CD4^+$ T_E express effector activity through the release of cytokines on TCR engagement (ie, recognition of specific peptide/MHC class II complexes on target cells).

Specific cytokines produced mainly by DCs at the time of T-cell activation can induce the differentiation of $CD4^+$ T cells into specialized subsets,

each of which produces a different constellation of effector cytokines. Interleukin-12 (IL-12) is primarily a product of activated DC and macrophages and exists as a heterodimer consisting of the p40 and p35 subunits. IL-12 interacts with its receptor on naive and activated $CD4^+$ T cells as well as $CD8^+$ T cells, signaling through signal transducer and activators of transcription 4 (STAT-4) to activate a number of genes. The outcome of this signaling process is to promote the differentiation of $CD8^+$ T cells into activated cytolytic effector cells and to drive the differentiation of $CD4^+$ T cells along a pathway leading to effector $CD4^+$ T cells capable of secreting IFN-γ on TCR engagement (ie, the generation of T_H1, T *helper* 1 effector T cells). The overall effect of IL-12 on T cells is to generate activated effector T cells that are efficient at viral clearance.

Interleukin-2 is a cytokine produced by both $CD4^+$ T cells and, to a lesser extent, $CD8^+$ T cells, during the initial phase of T-cell activation/differentiation. Engagement of the high-affinity IL-2 receptor (expressed only after T-cell activation) by IL-2 drives T-cell proliferative expansion. Although IL-2 was initially believed to be the primary T-cell growth factor (acting in an autocrine fashion to stimulate T-cell proliferation), T-cell activation/proliferative expansion occurs normally in the absence of IL-2 (eg, in IL-2–deficient patients or experimental animals with a targeted disruption of the IL-2 gene). Therefore, it appears that, in the absence of IL-2, other cytokines (eg, IL-15) can support the normal T-cell expansion in response to antigenic stimulation. As with many cytokines, IL-15 can be produced by a variety of cell types, both constitutively and in response to inflammation. IL-2 is of central relevance in the maintenance and function of regulatory T cells. A genetic deficiency in IL-2 results not in a defective T-cell response to antigen but in exaggerated T-cell proliferation (lymphoproliferative disease) and the development of autoimmune responses caused by a major functional defect in regulatory T cells.

A number of effector cytokines, including IFN-γ, IL-17A, and IL-22, are important in the context of containing viral infections. IFN-γ is produced primarily by activated $CD4^+$ T cell and $CD8^+$ T cells in response to TCR engagement and by NK cells after engagement of stimulatory NK cell receptors. The role of IFN-γ in augmenting antigen presentation (ie, upregulation of MHC class I and II expression, immunoproteasome subunit induction) has been discussed above. IFN-γ also plays a critical role in activating mononuclear phagocytes (macrophages) to destroy infected intracellular bacteria, and it presumably plays a similar role in the destruction of virus and, possibly, virus-infected cells when armed effector T cells respond to viral antigen at peripheral sites of virus infection. The early production of IFN-γ during the initiation of T-cell responses to antigen can drive T-cell differentiation to a type 1 (T_H1) pathway (characteristic of antiviral T-cell responses). The IFN-γ gene is rendered transcriptionally active by exposure of responding primary T cells to IL-12 resulting in IFN-γ production by activated effector T cells on subsequent TCR engagement by antigens and the induction of the T-bet transcription factor. IFN-γ ligates its receptor and induces the activation of STAT-1.

A combination of TGF-β, IL-6, and IL-23 contributes to the differentiation and maintenance of T_H17 cells, and these cells, which secrete large amounts of IL-17A and IL-22, are frequently induced during viral infections in parallel with the induction of T_H1 cells. IL-17A enhances the recruitment of neutrophils at sites of infection and IL-22 enhances barrier function at epithelial surfaces. T_H17 cells are therefore most functionally relevant in the context of clearing extracellular bacterial and fungal pathogens. Immune responses seen in viral infections are typically known as type I immune responses because they involve the activation of T_H1 and, activated $CD8^+$ T cells, NK cells and other type I innate lymphoid cells (ILC1s; which secrete IFN-γ). Type 3 innate lymphoid cells (ILC3s, that secrete IL-17A and IL-22) and T_H17 $CD4^+$ T cells may also frequently be activated.

While type I immune responses are important in the control of viral infections, type II responses are generally not of relevance in this context. The key $CD4^+$ T-cell subset that contributes to type II immunity is the T_H2 subset that secretes IL-4, IL-5, and IL-13. The induction of T_H2 $CD4^+$ T cells appears to play a pivotal role in the adaptive immune response to helminth (worm) and certain other parasitic infestations. T_H2 $CD4^+$ T_E are also now recognized to orchestrate allergic responses to non-pathogen–derived environmental antigens (eg, pollen, dander, dust mites) with development of IgE antibodies and eosinophil infiltration in response to these innocuous antigens. Exposure of responding $CD4^+$ T cells to IL-4 early in the activation/differentiation sequence is essential for the generation of T_H2 effector T cells in the SLO. The early low-level production of IL-4 by activated T cells before their commitment to T_H1, T_H2, or T_H17 differentiation may be sufficient to render the IL-4/5/13 gene locus capable of high-level IL-4, IL-5, and IL-13 production by $CD4^+$ T_H2 effectors following TCR engagement. The source of the IL-4 driving T_H2 cell differentiation is, however, still unclear. However, IL-4 produced by activated T cells, especially cells destined to become T follicular helper cells, serves both as a B-cell growth–promoting factor and also contributes to Ig class–switching during B-cell activation/differentiation (for certain isotypes). Engagement of the IL-4 receptor activates STAT-6 to mediate the effect of IL-4 on responding B and T cells. As will be discussed, IL-4 and IFN-γ act antagonistically to regulate T-cell differentiation and T-cell effector generation into T_H1 or T_H2 effectors.

Whereas virus infection, in general, drives $CD4^+$ T-cell differentiation toward the development of T_H1 and T_H17 $CD4^+$ T_E responses, apart from inducing T_{FH} cells, evidence indicates that, in certain chronic human infections, T_H2 $CD4^+$ T_E responses may predominate.[35,178] Because the induction of the T_H2 $CD4^+$ T_E response with IL-4 (and IL-10) production can suppress the development of a T_H1 $CD4^+$ T_E response characteristic of the adaptive immune response to virus infection, this form of virus-induced immune deviation could potentially result in the inhibition of virus clearance, leading to the development of persistent infection. However, states of induced dysfunction of $CD8^+$ T cells including $CD8^+$ T-cell exhaustion, discussed below, are likely far more relevant in the context of severe and chronic infections.

The Phenomenon of $CD8^+$ T-Cell Exhaustion/Dysfunction

Most viral infections of epithelial organs are cleared by virus-specific $CD8^+$ CTLs that induce the apoptotic death of infected cells. Clearly both severe acute as well as chronic viral infections have the potential to induce organ loss. It is in these contexts (as well as in the context of some antitumor $CD8^+$ T-cell responses) that $CD8^+$ T cells instead of becoming potent effectors are epigenetically altered and assume a terminally differentiated and dysfunctional state, anthropomorphically described as “exhaustion.” “Exhausted” $CD8^+$ T cells retain some function; they still kill infected target cells but less efficiently. So, dysfunctional “exhausted” T cells appear to have evolved to subserve two functions (1) to maintain an attenuated level of target cell killing that helps keep a viral infection partly in check but in so doing (2) to prevent organ loss. However, this phenomenon, with attendant immune dysfunction, does result in chronic infection, viral persistence, immunopathology, and the failure to eliminate certain antigenic tumors.

Exhausted $CD8^+$ T cells are induced by chronic antigen stimulation and likely the milieu of severe inflammation as well, though that is less well defined. While PD-1–mediated inhibitory signaling is required in exhausted T cells, PD-1 itself is expressed on most activated $CD4^+$ and $CD8^+$ T cells and cannot on its own define the exhausted state. The process of exhaustion is a continuum. Exhausted/dysfunctional $CD8^+$ T cells exist broadly in two forms within the continuum. These include initially generated self-renewing "stemlike" progenitor PD-1^{Hi} TCF-1^+ cells that have some features of central memory cells and express high levels of the PD-1 inhibitory receptor. These cells are still plastic and "rescuable," but they give rise to terminally differentiated exhausted/dysfunctional $CD8^+$ T cells that have been molded by epigenetic remodeling into an irreversibly dysfunctional state and can no longer be rescued. These terminally exhausted T cells express both the PD-1 and TIM-3 inhibitory receptors, are no longer dependent on the TCF-1 transcription factor, and instead express TOX-1.

The PD-1 inhibitory receptor contains cytosolic tyrosine-based ITSM (immunoreceptor tyrosine–based switch) and ITIM (immunoreceptor tyrosine–based inhibitory) motifs. Ligation of PD-1 by its ligands, PD-L1 or PD-L2 (typically expressed on DCs but also induced on some tumors) induces the tyrosine phosphorylation of the ITSM and the recruitment of SHP-2, which can dampen T-cell activation. PD-1 is clinically considered as an "immune checkpoint" molecule. PD-1 blockade by therapeutic antibodies has become a major, often "curative," therapy in some antigenic cancers. Though checkpoint blockade can reverse chronic infection in animal models, the risk of adverse effects, even possible organ loss, has so far tempered its clinical use in chronic viral infections.

Regulatory T-Cell Function in Antiviral Adaptive Immune Responses

Homeostasis in the immune system depends on a balance between the responses to control viral infection and the reciprocal responses to prevent excessive immune responses. Regulatory T cells (T_{reg}) have been reported to play a crucial role in suppressing immune responses to self-antigen and in preventing autoimmune diseases. T_{reg} also control immune responses to pathogens, including viruses. T_{regs} are categorized as naturally occurring, thymus-derived T_{reg}, or induced, peripheral T_{reg}, which are particularly abundant in the gut and the placenta.

There is some knowledge regarding the suppressive function of T_{reg} cells. Suppressive activity by T regs and other inhibitory $CD4^+$ T cells is broadly mediated by secretion of immunosuppressive cytokines (ie, IL-10, transforming growth factor β [TGF-β]) or by CTLA-4–mediated capture and degradation of B7 on DCs following cell-cell contact. IL-10 inhibits immune responses in many ways including the suppression of TNF and IL-12 production by DC and macrophages; TGF-β also inhibits immune responses in numerous ways including attenuation of the induction of the transcription factor, T-bet that is relevant in T_H1 $CD4^+$ T cells, activated $CD8^+$ T cells, and NK cells for IFN-γ production especially in the context of viral and other intracellular infections. In addition, TGF-β produced by T_{reg} cells induces IL-10 production by T_H1 cells through SMAD-induced activation of the IL-10 promoter. Given the effect of IL-10 and T_{reg} cells on the inhibition of the expression of MHC class II and costimulatory molecules by DC, it is likely that cytokine-mediated suppression might occur at the level of APC. CTLA-4 present at high levels on $CD4^+$ $CD25^+$ T_{reg} cells also functions at the APC level; it has been shown to impair the provision of signal 2 for T-cell activation in SLOs, since CTLA4 can capture and remove B7 from DCs by a process called transendocytosis.

T_{regs} and other inhibitory cytokine producing T cells have been implicated in the suppression of cellular immune responses directed against pathogens, including chronic viral infections. IL-10–producing T_{reg} cells have been shown in several chronic infections (eg, HSV, EBV, HCV). EBV infection is associated with the induction of T_{reg} cells specific for latent membrane protein 1 (LMP1) during the lytic cycle. In case of HCV infection, HCV-specific CTL traffic to the liver produces IL-10 and reduces liver inflammation. In addition, the frequency of $CD4^+$ $CD25^+$ T_{reg} cells is greater in chronically infected patients than in recovered or healthy patients,[17,34,139,182] suggesting that T_{reg} cells play a role in HCV persistence. Apparently, suppression mediated by $CD4^+$ $CD25^+$ T cells isolated from patients chronically infected with HCV is not cytokine-mediated, because neutralizing antibodies to either IL-10 or TGF-β do not reverse suppression.[17] The presence of $CD4^+$ $CD25^+$ T_{reg} and their suppression of effector T cells from patients with HCV indicate that these T_{reg} cells play a role in HCV persistence. It was also found that the frequency of $CD4^+$ $CD25^{hi}$ T cells in patients with hepatocellular carcinoma is greater than in individuals chronically infected with HCV.[139] Importantly, the reduced number of $CD4^+$ $CD25^+$ T_{reg} in patients infected with HCV is associated with the development of autoimmune disease such as cryoglobulinemia characterized by B-cell proliferation and autoantibody production. This provides compelling evidence that T_{reg} cells play a dual role in viral infection: (1) prevention of viral clearance and (2) the regulation of autoimmunity.

Even though T_{reg} cells might be involved in suppression of host immune responses to viral infection, immune responses to viral infection can nevertheless result in host tissue damage in many virus-mediated diseases. Thus, immunoregulatory mechanisms (including the induction of T_{reg} cells) are paradoxically essential to control virus-induced immunopathology. Direct evidence of a role for T_{reg} cells in preventing immunopathology has come from studies using mouse models of viral infection. Theiler's murine encephalomyelitis virus (TMEV) infection induces $CD4^+$ T-cell–mediated demyelinating disease.[76] TMEV-specific $CD4^+$ T-cell blasts transferred into susceptible, irradiated C/cByJ mice accelerate clinical disease and enhance TMEV-specific, delayed-type hypersensitivity (DTH) and proliferation in these animals. Significantly, $CD8^+$ T cells from infected C/cByJ mice suppress the *in vivo* disease progression as well as virus-specific, T-cell responsiveness in recipients of TMEV-specific $CD4^+$ T-cell blasts. The transfer of virus-specific $CD8^+$ T_{reg} cells at the time of Theiler's virus infection has been shown to reduce inflammation by preventing the pathogenic role of $CD4^+$ T cells. In case of HSV infection, depletion of $CD4^+$ $CD25^+$ T cells before viral infection increases the generation of virus-specific $CD8^+$ T-cell responses and viral clearance.[188,189] In contrast, the severity of T-cell–mediated lesions in the cornea of HSV-infected mice were increased on removal of $CD4^+$ $CD25^+$ T cells. Thus, $CD4^+$ $CD25^+$ T_{reg} cells are likely involved in reducing the severity of immune-mediated inflammatory lesions by preventing the pathogenic effects of $CD4^+$ T_H1 cells, as well as limiting the migration of these cells to inflammatory sites. During chronic viral infections, T_{reg} cells might be beneficial to the host by maintaining a balance between efficient effectors and memory responses, but with a low level of inflammation to limit host tissue damage. Understanding how T_{reg} induction occurs during infection could be helpful for designing therapeutic intervention against chronic infection.

Adaptive Immune Memory

Two features define the adaptive immune system and distinguish it from the innate immune system are (1) exquisite specificity for antigen and (2) the establishment of immunologic memory. Immunologic memory is perhaps the most important consequence of the induction of an

adaptive immune response. As a result of clonal expansion of antigen-specific B or T lymphocytes, and a lower activation threshold for memory cells, the cellular immune system can respond more rapidly/efficiently to previously encountered pathogens. The capacity of B and T cells to adapt to a previously encountered antigen, together with circulating antibodies, is the basis for vaccination. Memory B and T cells generated after infection or vaccination are generally long-lived. In humans, memory T-cell responses (or circulating antibody titer) can be detected decades after pathogen exposure, under conditions where subsequent reexposure to (subclinical infection with) the organism or persistent infection is unlikely. Memory B and T cells directed to an antigen are usually present at a frequency 100-1000 times higher than the corresponding naive immune cell precursors of memory T and B cells. The factors controlling the formation/duration of the memory immune responses are not yet well understood, but several cytokines may play an important role in maintaining the viability and basal (homeostatic) proliferation of memory cells.

B-Cell Memory

The phrase "B-cell memory" is often equated with long-term humoral immunity.[6,9,37,102,107,202] Two principal mechanisms exist: (1) specific memory B cells expressing BCR to viral antigens, so that viral infection leads to rapid activation of specific B cells and ultimately the production of high-affinity, class-switched antibody to virus and (2) preexisting serum and tissue high-affinity, class-switched antibody to virus from long-lived plasma cells in the bone marrow.

Memory B Cells

Memory B cells[6,37,60,102,103,169] express class-switched, somatically mutated surface antibody and can be detected as antigen-specific cells using labeled antigen and a technique such as flow cytometry. They are in a resting state and do not secrete antibodies unless stimulated by antigen. When this stimulation is carried out *in vitro*, the memory B cells can be readily detected by enzyme-linked immunospot (ELISPOT) assays. In mice, multiple subtypes of memory B cells have been defined by a number of cell surface markers and gene activation markers. After clearance of virus on first exposure, memory B cells accumulate in the spleen and other lymphoid tissues and circulate in the blood. Reexposure to virus leads to rapid secondary antibody responses to viral antigens. This response arises because of rapid proliferation and differentiation of memory B cells in a $CD4^+$ T-cell–dependent manner. The specific B-cell population expands massively and differentiation to plasmablasts generates a burst in specific antibody production and in antibody levels. Thus, repeated exposure to antigen is one way in which memory B-cell levels can be maintained.

Although controversies have existed over the years, it appears that memory B cells can be long-lived even in the absence of antigen. An elegant experiment by Maruyama et al.[123] is persuasive. Mice were engineered so the memory B cells expressing a BCR against antigen-1 could be switched *in vivo* to express a BCR specific for antigen-2. B cells expressing BCR to antigen-2 were identified as persisting in the mice as well as B cells expressing BCR to antigen-1, although the mice had never encountered antigen-2. It should also be noted that BCR expression is required for B-cell survival[106] so the possibility exists that nonspecific BCR stimulation, perhaps via a low-affinity antigen interaction, is needed for persistence of B-cell memory. Alternatively, the BCR may receive a signal from another molecule such as a cytokine or a molecule such as a B-cell–activating factor (BAFF).

One interesting phenomenon related to memory B cells is that of *original antigenic sin* (OAS),[135,195,212] or, closely related, "imprinting".[126,163] OAS was first proposed to describe a phenomenon whereby an individual, originally infected with a virus and then later with variant of this virus, makes an antibody response during the second viral infection that reacts more strongly to the original virus than the newer variant.[48] Boosting of memory B cells against the first virus dominates activation of naive B cells to the second virus, resulting in a condition whereby specific antibody responses to the second virus will not be as great compared with a naive host that has never encountered the first virus. OAS has been reported in both humans and animals and against a range of viruses including dengue virus, influenza virus, Japanese encephalitis virus, West Nile virus, Zika virus, and enterovirus. OAS presents a challenge to the design of vaccines for viruses that exist as diverse variants in the population, most notably for influenza.[58,126]

Long-Lived Plasma Cells

Plasma cells are terminally differentiated, nondividing cells able to secrete relatively large amounts of antibody.[1,39,47,88,89,115,161,190,191] At one time, the dogma was that plasma cells were short-lived, although some reports had described longer-lived plasma cells. It now appears clear, however, that two populations of plasma cells exist. Short-lived plasma cells (plasmablasts) survive for only a few days, produce antibody in extrafollicular foci, and are probably crucial in the very early response to pathogen. In mice, it has been shown clearly that the second population of plasma cells, secreting high-affinity antibody, is long-lived. Irradiation experiments have shown that the half-life of plasma cells secreting antibody to LCMV was 94 days in the bone marrow and 172 days in the spleen.[175] Pulse chase experiments showed that plasma cells secreting specific antibody to ovalbumin survived without cell division for a minimum of 3 months.[120] These half-lives constitute a sizable proportion of the total lifespan of the mouse. Plasma cells likely live for many years in humans and thereby contribute to the maintenance of antibody levels over decades.[66] The bone marrow niche appears to provide survival signals to plasma cells, because *in vitro* such cells survive for only a few days. It has been questioned whether plasma cells could survive for decades in humans and one possibility is that plasma cells are being periodically replenished from specific memory B cells by bystander activation and differentiation.

Long-term Humoral Immunity

Humoral responses can be measured in the sera of humans many years after the last known contact with the pathogen (Table 10.6). Similarly, many vaccines induce serum antibody responses that are present decades later.[150] A key question has been: are these responses maintained by intrinsic mechanisms or is there a periodic boosting of immunity through contact with antigen? This question can be very difficult to answer given the unknowns associated with typical human contacts with pathogens. Studies,[45] however, suggest, for at least two vaccines, that serum antibody levels are maintained in the absence of antigen boosting.

TABLE 10.6 Humoral response to acute viral infection in humans

Example	Virus Family	Persistence of Antibody
Systemic infections		
Chikungunya	*Alphaviridae*	30 y
Rift Valley fever virus	*Bunyaviridae*	12 y
Dengue	*Flaviviridae*	32 y
Yellow fever	*Flaviviridae*	75 y
Measles	*Paramyxoviridae*	65 y
Mumps	*Paramyxoviridae*	12 y
Polio	*Picornaviridae*	40 y
Hepatitis A	*Picornaviridae*	25 y
Smallpox	*Poxviridae*	40 y
Vaccinia	*Poxviridae*	75 y
Rubella	*Togaviridae*	14 y
Mucosal infections		
Coronavirus	*Coronaviridae*	12 mo
Influenza virus	*Orthomyxoviridae*	30 mo
RSV	*Paramyxoviridae*	3 mo
Rotavirus	*Reoviridae*	12 mo

Modified from Slifka MK, Ahmed R. Long-term humoral immunity against viruses: revisiting the issue of plasma cell longevity. *Trends Microbiol*. 1996;4(10):394-400. Copyright © 1996 Elsevier, with permission. Ref.[174]

A large cross-sectional study of poliovirus immunity in Sweden, a country from which the virus has been eradicated and which only used inactivated poliovirus as a vaccine, showed substantial antipoliovirus antibody titers in all age groups.[20] Virtually no differences in titers were found in the different age groups, indicating the maintenance of antibody titers over decades in the absence of further vaccination or exposure to live virus. Notably, in the same study, declining antibody titers against tetanus and diphtheria were observed.

Two studies have looked at smallpox vaccination using vaccinia virus.[46,72] Vaccinia is an excellent model for investigating immune memory because the virus is typically cleared from the site of infection within a month, does not persist, and does not spread systemically in healthy individuals. Furthermore, smallpox was declared eradicated in 1980, and vaccinia immunization was discontinued in the United States in 1972. Nevertheless, specific memory B cells could be detected in U.S. individuals more than 50 years after their last vaccination.[45,46] Further, memory B cells seemed to show a bimodal kinetics. A drop from peak responses occurred at vaccination to an ~10-year time point, but stable levels were seen between 10 and 60 years. Serum antibody responses could be identified at 60-75 years after vaccination.[46,57,72] Because overall antivaccinia antibody levels correlated well with neutralizing antivaccinia antibody levels, it was postulated that different specificities may be equally well preserved. Further, the results suggest, as originally posited by Jenner more than 200 years ago, that protective immunity against lethal smallpox infection may be lifelong.[173]

Finally, it is apparent from Table 10.6, that mucosal antibody responses are much shorter-lived than serum responses. This may indicate that plasma cells initially produced at mucosal sites migrate to the bone marrow and contribute less to mucosal antibody production.

T-Cell Memory

Unlike the Ig receptors on B cells, the genes encoding the TCR on $CD4^+$ T cell and $CD8^+$ T cells do not undergo any additional somatic mutations and memory T cells do not, therefore, exhibit affinity maturation after antigen encounter. Memory T cells distinguished functionally from their naive precursors by being present at increased frequency (a result of clonal expansion of naive antigen-specific T cells) and by their lower activation threshold (ie, decreased requirement for CD28 costimulation) to trigger cell proliferation/differentiation. Certain memory T cells can also rapidly express effector activity (eg, proinflammatory/antiviral cytokines, such as IFN-γ and TNF-α) within hours after TCR engagement on these T cells and without DNA synthesis (additional cell proliferation).

Naive T-cell activation/proliferation and memory T-cell population formation/maintenance are controlled by three cytokines: IL-2, IL-7, and IL-15. The receptors on T cells for these three cytokines (and for a few others including IL-4, IL-9, and IL-21) have a common feature—these heterodimeric and trimeric receptors have a common signaling subunit, the so-called *common γ chain* (γ_c), which participates in both ligand (cytokine) binding and Janus kinase (JAK)/STAT-dependent signal transduction. IL-2 and IL-15 can drive proliferation of activated naive T cells after TCR engagement and IL-4, which engages another member of the γ_c family of cytokine receptors, will also support proliferation of differentiating T_H2 $CD4^+$ T cells. Current evidence suggests that, once memory T cells are formed, they can undergo basal/homeostatic low-level proliferation, which is IL-15–dependent, and long-term memory T-cell viability (ie, suppression of apoptosis) is supported by IL-7, which

upregulates/sustains the expression of antiapoptotic Bcl-2 gene family members in developing memory T cells.

Although activated naive T cells give rise to both effector (T_E) and memory (T_M) T cells, the relationship of the two T-cell subtypes is unclear and it is still debated whether T_E give rise to T_M in a linear progression or if T_E and T_M are separate cellular subsets. T_M can be distinguished from naive T cells and, to a lesser extent, from T_E by the expression of certain cell surface molecules (eg, isoforms of CD45) differentially expressed on T_M and naive T cells. Several lines of recent evidence indicate that a further subdivision may exist of $CD4^+$ T-cell and $CD8^+$ T-cell T_M into central memory (T_{CM}), peripheral/effector memory (T_{EM}), and tissue-resident (T_{RM}) memory populations. T_{CM} express cell surface molecules such as CD62L and the chemokine receptor CCR7, which facilitate the trafficking of T_{CM} from the blood into the SLO (particularly the LN). So, T_{CM} mimic the recirculation pattern of naive T cells (blood 2799 → SLO → blood) and respond to antigen delivered to the SLO; and, as occurs with naive T cells, T_{CM} may therefore require both activation and proliferation to express effector activity. By contrast, T_{EM} do not display the homing receptors for the SLO and have been suggested to reside primarily in the blood and peripheral tissues where they can rapidly respond to a pathogen at the initial site of pathogen entry in the periphery. Unlike T_{CM}, T_{EM} appear to undergo limited proliferation in response to antigen but rapidly express antimicrobial effector activity in response to infection. T_{CM} and T_{EM} are maintained by a stemlike memory population that expresses the TCF-1 transcription factor. Some memory cells enter peripheral tissues and reside there, undergoing self-renewal at these sites. These cells constitutively express CD103 and CD69 and are important for clearing viruses in some peripheral organs. Although these classifications of memory T cells based on cell surface markers are useful, evidence from the analysis of the immune response to virus infection in humans suggests that considerable heterogeneity exists in cell surface marker expression on circulating T cells in the peripheral blood. Thus, under conditions of natural infection (and possible vaccination), the memory T-cell response may consist of a continuum of activation/differentiation states—at least, based on activation/differentiation marker expression.

Viral Strategies to Evade the Adaptive Immune Response

Immune responses to viruses involve complex molecular and cellular interaction between the virus and its host. Thus, any stage in this interaction could be targeted by a pathogen and used for its own benefit. The systematic study of viral genomes reveals that most viruses have evolved some means of escaping or subverting immune defenses and that some viruses have many genes devoted to this purpose. Whereas the large, more complex DNA viruses encode viral proteins to avoid immune recognition, RNA viruses usually encode smaller numbers of proteins compared with DNA viruses with large genome materials. Regardless of virus type (DNA or RNA), it seems that many, and possibly all, pathogens that cause chronic infections have evolved strategies to subvert the immune responses of the host (Table 10.7). These strategies include evasion of humoral and cellular immunity by antigenic variation, interference with antigen processing and presentation, and modulating the production of cytokines. Understanding the complex interactions between the immune system of the host and the invading viral pathogen will eventually enable researchers to devise better strategies for preventing viral diseases.

TABLE 10.7 Viral strategies to avoid host adaptive immunity

Escape by mutations
Escape by latency
Escape by destruction of immune cells
Escape by subverting antigen processing and presentation
Inhibition of T-cell–mediated target cell lysis
Interference of cytokine function

Viral Evasion of Humoral (B-Cell) Immunity

Viruses use a number of mechanisms to evade humoral immune responses and viral neutralizing antibodies in particular (reviewed in Refs.[30,110,133,172]). Viral strategies to evade neutralizing antibody responses to the viral surface proteins include (1) mutation of surface proteins by antigenic drift and shift, and recombination, most notably for highly antigenically variable viruses such as influenza virus, HIV, and HCV; (2) masking of conserved epitopes; (3) glycan shielding as seen for HIV and Ebola viruses, for example; (4) surface protein decoys that may “mop up” neutralizing antibodies under certain conditions, for example, as with HBV; (5) original antigenic sin or imprinting; (6) irregular surface protein spacing that tends to reduce immunogenicity; and (7) multiple surface proteins and virus forms, for example, poxviruses encode multiple proteins for entry. Viruses can also evade humoral responses by spreading directly between cells, upregulating and hijacking host complement regulatory proteins, encoding antagonistic Fc and complement receptors, and inducing immunosuppression.

Viral Evasion of Cellular (T-Cell) Immunity

Viruses have multiple strategies to subvert T-cell responses. Two well-characterized subversion mechanisms (ie, inhibition of antigen processing/presentation and interference with cytokine function) are highlighted below.

Escape by Subverting Antigen Processing and Presentation

A number of viruses have devised strategies to impair presentation of their antigens by the MHC class I pathway, thus reducing activation of antigen-specific $CD8^+$ T cells as well as recognition of virus-infected cells by $CD8^+$ T cells. This stresses the importance of this cell subset for antiviral defense. Nearly every step of the MHC class I presentation pathway can be interfered with, and some viruses encode multiple proteins that act at different levels of the MHC class I–processing pathway (Table 10.8): (1) inhibition of MHC class I synthesis, (2) antigenic peptide generation, (3) transporter associated with antigen processing, and (4) the cell surface expression of MHC class I molecule expression. Although interference of the MHC class II antigen–processing pathway by virus is less common, however, some evidence indicates that viruses are able to inhibit this pathway.

TABLE 10.8 Inhibition of antigen presentation via the major histocompatibility complex (MHC) class I pathway

Steps to Interfere With MHC Class I Pathway	Virus-Encoded Proteins
Inhibit MHC class I synthesis	Lentivirus (Vpu), SARS-CoV-2 ORF-6
Inhibit transporter associated with antigen processing (TAP)	
• Expression	EBV (vIL-10), HCMV (UL111A)
• Function	HCMV (US6), HSV (ICP47)
Inhibit MHC class I transport	
• Retain MHC class I in the endoplasmic reticulum (ER)	HCMV (US3), adenovirus (E3-19K)
• Retain MHC class I in the pre-Golgi compartment	MCMV (m152)
• Dislocate MHC class I to the cytoplasm	HCMV (US11, US2)
• Dislocate MHC class I to lysosomes	MCMV (m6/gp48), SARS-CoV-2 ORF-8
• Bind to cell surface MHC class I molecules	MCMV (gp34)
• Increase endocytosis of MHC class I molecules	HIV (nef), HHV-8 (K3, K4)

EBV, Epstein-Barr virus; HCMV, human cytomegalovirus; HSV, herpes simplex virus; MCMV, murine cytomegalovirus; HIV, human immunodeficiency virus; HHV, human herpesvirus.

Inhibition of Class I or II Major Histocompatibility Complex Synthesis

Viral proteins, including HIV Tat protein, have been reported to suppress/inhibit MHC gene promoter activity.[85] It has been shown for HIV Tat protein suppression that the activity of the MHC class I gene promoter was decreased up to 12 times. The SARS-CoV-2 ORF6 protein interferes with type II interferon–meditated STAT1 activity, thus limiting the induction of NLRC5 an MHC class I gene transactivator.[213] ORF6 also separately interferes with the nuclear import of NLRC5.[213] In respect to the inhibition of MHC class II expression, HCMV can impair MHC class II expression through two distinct pathways. MHC class II molecules are expressed only on a subset of cells, and this expression is regulated at the transcriptional level through control elements that include those that allow both constitutive and cytokine-induced transcription of the MHC class II genes. The MHC class II transactivator (CIITA) is essential for constitutive and induced transcription and is the rate-limiting factor of MHC class II production. One of the four promoters controlling CIITA production is activated by IFN-γ, which, on binding to its receptors, triggers through a Janus kinases signaling cascade, leading to transcription of IFN-inducible genes. The inhibition of IFN-γ–stimulated MHC class II expression occurs by two different mechanisms: (1) inhibition of JAK/STAT signaling[132] and (2) repression at the CIITA promoter.[111]

Inhibition of Antigenic Peptide Generation

The metalloproteases, CD10 (endopeptidase) and CD13 (aminopeptidase N), are downregulated during HCMV infection.[149] CD10 expression is apparently blocked at the transcriptional or translational level, whereas CD13 seems to be retained within the ER compartment. These endopeptidases play a role in peptide processing in both MHC class I and II antigen presentation pathways. Both CD10 and CD13 peptidases are expressed on the cell surface and trim antigenic peptides to a size that allows their binding to the groove of class I or II molecules.

Inhibition of Peptide Transport

Many of the strategies used by viruses to inhibit the transport of antigenic peptides are reviewed elsewhere.[116] The peptide transporter TAP is needed to shuttle peptides from the cytoplasm to the ER, in which they associate with MHC class I determinants. TAP peptide transport is inhibited by a protein of HCMV, US6, which acts from the luminal site of the ER. HCMV US6 protein specifically binds at the ER-luminal loops of TAP signals across the membrane to the nucleotide-binding domains. This binding prevents ATP hydrolysis of TAP, which is necessary for peptide transport.

The ICP47 polypeptide from HSV inhibits TAP by binding to TAP's peptide-binding site, thus preventing its association with other peptides. This results in an insufficient number of peptides in the ER for binding to MHC class I molecules. In contrast, TAP-independent peptide loading of MHC class I molecules is not affected by US6 or ICP47. As described below, EBV encodes a protein that weakly binds the IL-10 receptor. As with cellular IL-10, the EBV protein reduces expression of TAP and of MHC class II.

Nontranscriptional Inhibition of Major Histocompatibility Complex Class I Cell Surface Expression

Modulation of MHC class I expression has been reported in a number of viral systems including adenoviruses, HCMV, HSV, HIV, and SARS-CoV-2. HCMV US3 protein, a type I membrane protein, prevents maturation of MHC class I molecules by retaining them in the ER compartment. US3 protein binds to MHC class I molecules in a transient fashion but retains them efficiently in the ER. The US3 luminal domain is responsible for ER retention of US3 itself, whereas both the US3 luminal and transmembrane domains are necessary for retaining MHC class I in the ER. In contrast to HCMV US3 protein, HCMV US2 and US11 catalyze the dislocation of MHC class I products, resulting in their rapid degradation. US11 protein uses its transmembrane domain to recruit MHC class I products to human homologue of yeast Der1p, a protein essential for the degradation of MHC class I molecules catalyzed by US11, but not by US2. HCMV US2 has been shown to differentially affect surface expression of MHC class I but only targets membrane-bound (not soluble) HLA-G1 antigen for degradation.

Adenovirus expresses a protein E3/19K, which is an ER-retained protein; it has a cytosolic ER retention signal in the C-terminus of its cytoplasmic tail (–DEKKMP). The 19K binds certain murine MHC class I molecules (eg, A2, K^d, L^d, but not K^k, or D^d) and holds them in the ER. This results in decreased MHC class I expression and decreased CTL recognition. An immediate early gene product of HSV, the ICP47 gene product, interferes with transport of peptides from the cytoplasm to the ER, thereby depriving MHC class I molecules in the ER of peptides. This results in both the ER peptides and destabilization and retention of MHC class I molecules in the ER. The SARS-CoV-2 ORF8 protein reduces host MHC class I expression protein expression by targeting MHC class I protein for autophagy and lysosomal degradation.[218]

Interference of Cytokine Functions

Cytokines (eg, IFN and TNF) induce intracellular pathways that activate an antiviral stage or apoptosis and thereby limit viral replication. A large number of cytokines induce mechanisms that enhance immune recognition and/or immune responses to protect against viral infection. In addition, antiviral cytokines play a pivotal role in removal of infected cells by NK cells or CTL. Thus, it is not surprising that viruses use immune evasion strategies to control the function of cytokines.

Recently, viral modulation of cytokines by several viruses has been reported. Not included here are descriptions of the ability of viruses like SARS-CoV-2 and Ebola among many others to compromise the production of type I interferons—those issues are considered in Chapter 8. Three different mechanisms have been shown to affect the activity of cytokines more relevant to adaptive immunity (Table 10.9). Viruses produce cytokine homologues, which bind to the same cytokine receptors. Alternatively, viruses generate antigens that mimic cytokine receptors and, thus, neutralize the corresponding factors. Finally, they can directly interfere with the action of cytokines by generating soluble cytokine-binding proteins. The function of viral homologues of cytokine and their receptors are diverse. The virus-encoded cytokine homologs might be involved in inactivating inflammatory cytokines or redirecting the immune responses.

TABLE 10.9 Viral modulation of the cytokine system

Interrupt cytokine production
• Interfere with cytokine and chemokine synthesis
• Inhibit the generation of functional cytokines
Interfere with cytokine action
• Encode homologs of cytokines and cytokine receptors
– Type I interferon (IFN) homolog: VV (B18R)
– IFN-γ homolog: VV (B8R)
– Interleukin (IL)-6: KSHV (K2)
– IL-8 homolog: HCMV (UL146, 147)
– IL-10 homolog: EBV (BCRF1), HCMV (UL111A)
• Generate soluble cytokine receptors to neutralize cytokines
– IFN-γ receptor: myxoma virus (MT-7)
– IL-1βR: VV WR (B15R)
– TNFR homolog: orthopoxvirus (crmB, crmD)
Interfere with cytokine effector function
• Alter cytokine signaling pathway

HCMV, human cytomegalovirus; EBV, Epstein-Barr virus; TNFR, tumor necrosis factor receptor.

Inhibition of cytokine production has been shown by poxvirus; cowpox virus (CPV) cytokine response modifier (CrmA) protein inhibits the production of caspase-1, which prevents the proteolytic cleavage of prointerleukin—1β (pro–IL-1β) to mature IL-1β. In addition, the attachment of measles virus to its cellular receptor, CD46, a complement-regulatory protein, inhibits the production of IL-12. Many virus-encoded mechanisms are involved in blocking the effector functions of cytokines, such as the antiviral state induced by IFN or apoptosis triggered by TNF, and intracellular antagonists of TNF or IL-1/Toll-like receptor signaling. Interestingly, LMP1 of EBV recruits components of tumor necrosis factor receptor (TNFR) and CD40 signal transduction machinery, which induces biological responses to help viral replication.

Receptor Binding and Modulation of Immune Cell Function

Measles virus binds to, and enters, monocyte/macrophages using CD46 molecule (a C3b receptor) as its receptor for entering cells; cross-linking CD46 inhibits the production of IL-12 by macrophages. Therefore, measles-induced immune suppression may be related to the failure of infected APC to make the proinflammatory cytokine IL-12. Another IL-12 homolog, UL111A, is encoded by HCMV.

Cytokine and Cytokine Receptor Homologs

Alterations in cytokine function represent a newly appreciated mechanism of evasion. One of the most striking examples of this mechanism has been reported in the EBV. EBV has apparently cannibalized the gene for the human IL-10 cytokine. The viral genome, gene BCRF1, encodes a protein with 70% amino acid homology to human IL-10. IL-10 inhibits cytokine production by T_H1 type T cells and monocytes (macrophages) through its action on macrophage function. IL-10 also activates B lymphocytes and upregulates BCL-2. The viral IL-10 has all of the same activities as human IL-10.

The understanding the role of viral immunomodulatory proteins in the context of infection is limited, mainly because of the lack of appropriate experimental models of infection. In many cases, we can only predict the *in vivo* function of a viral protein in view of a known function of cytokines. As expected, inactivation of poxvirus-soluble cytokine receptors (eg, the myxoma virus vTNFR and vIFN-γR, or the vaccinia virus vIFN-α/βBP) leads to virus attenuation. The attenuated phenotype of the myxoma virus vIFN-γR (M-T7) mutant might also be attributed to the ability of M-T7 virus to bind chemokines, which is consistent with increased leukocyte recruitment to sites of infection. The consequences of the inactivation of a viral immunomodulatory protein on the outcome of infection, however, might be unpredictable. Deletion of vaccinia vIL-1βR exacerbates vaccinia virus infection in a mouse intranasal model because of enhanced systemic activity of the proinflammatory cytokine IL-1β, which might cause increased fever and weight loss, leading to enhanced mortality of the infected host. This indicates that some viral immunomodulatory proteins might downregulate immune-mediated pathology to favor equilibrium with the host, rather than to increase viral replication.

Virus-Induced Immune Dysregulation and Autoimmune Disease

Many chronic diseases can occur when the immune system is active, often causing the affected tissues to be inflamed and abnormally infiltrated by lymphocytes and other leukocytes. No active infection is associated with these diseases, however. Thus, these diseases are caused by the immune system itself, which attacks cells and tissues of the body. Chronic diseases of this kind are known as *autoimmune disease* because they are caused by immune responses directed toward self-components of the body. Several genetic risk factors and protective elements have been shown to influence the susceptibility of autoimmune disease. A considerable discordance in incidence of autoimmune disease comparing identical twins indicates, however, that in many cases, additional factors such as environment modulators could influence the incidence of autoimmune diseases.

Indeed, viral infections, particularly chronic viruses, are shown to enhance autoimmune disease in susceptible individuals, because infections frequently induce strong inflammatory responses in various organs.[138,141] Several major pathways exist through which viruses can initiate or more likely modulate autoimmunity. First, direct infection of target cells/organs can cause the release of sequestered autoantigens and enhance antigen presentation. Second, local inflammation might alter the repertoire of self-epitopes presented by APC by altering the antigen degradation properties of the proteasome. Third, presentation of pathogen epitopes with structural or sequential similarity to self-epitopes might specifically activate autoreactive lymphocytes. Some viral infections, however, can also act to ameliorate autoimmunity. Several reports support the association of autoimmune diseases with viral infection.[16,61,108,129,181,217] Indeed, associations with infectious agents have been suggested for a multitude of autoimmune diseases, including type 1 diabetes (TID), multiple sclerosis (MS), and ankylosing spondylitis. Attempt to establish a direct epidemiologic, statistically relevant association between microbial infections and various autoimmune disorders, has been unsuccessful so far.

Hepatitis C virus has been reported to be involved in the cause of autoimmune disorders, including myasthenia gravis, autoimmune hepatitis, cryoglobulinemic vasculitis, and rheumatoid diseases. It has been also speculated that serious liver injury occurs in chronic HCV infection, and autoimmune mechanisms may contribute to hepatic destruction.[112] Anti-liver–kidney microsome type 1 (LKM-1) autoantibodies directed against the cytochrome P450 2D6 (CYP2D6) are considered specific markers of type 2 autoimmune hepatitis.[119,211] Interestingly, LKM-1 autoantibodies are also found in 5% of sera from patients chronically infected by HCV. Molecular mimicry between HCV proteins and CYP2D6 has been proposed to explain the emergence of these antibodies. Indeed, recent studies revealed cross-reactivity caused by molecular mimicry at the B-cell level between CYP2D6 and HCV NS3, NS5a proteins.[122] This might explain the presence of anti-LKM in patients with chronic HCV.

Epstein-Barr virus has been linked with chronic autoimmune disease (eg, MS or systemic lupus erythematosus [SLE])[67,91] because self-reactive B lymphocytes are easily susceptible to EBV infection. Recent studies have strengthened the epidemiological link between EBV infection and MS.[16] Additionally, in mechanistic studies, antibodies that are generated against EBNA-1 cross-react with GlialCAM in the brain and spinal cord in MS patients, resulting in an autoimmune response derived from molecular mimicry.[108] Studies analyzing changes in viral persistence in SLE provide compelling evidence that EBV infection is perturbed in the development of SLE. The increased viral load is caused by an increase in the numbers of latently infected cells, particularly in patients with active disease. In addition, patients with SLE have aberrant expression of viral latent and lytic genes in the blood. This suggests that immune dysfunction in SLE alters the regulatory mechanisms of EBV persistence such that B-cell dysregulation might be involved in viral persistence. More thorough and complete studies on changes in viral persistence in SLE would help in the understanding of the nature of EBV-mediated immune perturbation and further provide new insight into the mechanisms involved in regulating the virus.

REFERENCES

1. Aaron TS, Fooksman DR. Dynamic organization of the bone marrow plasma cell niche. FEBS J. 2022;289(14):4228-4239.

2. Abbas AK, Lichtman AH, Pillai SP. Cellular and Molecular Immunology. 10th ed. Elsevier; 2021.

3. Abbott RK, Lee JH, Menis S, et al. Precursor frequency and affinity determine B cell competitive fitness in germinal centers, tested with germline-targeting HIV vaccine immunogens. Immunity. 2018;48(1):133-146 e6.

4. Abela IA, Berlinger L, Schanz M, et al. Cell-cell transmission enables HIV-1 to evade inhibition by potent CD4bs directed antibodies. PLoS Pathog. 2012;8(4):e1002634.

5. Abernathy ME, Dam KA, Esswein SR, Jette CA, Bjorkman PJ. How antibodies recognize pathogenic viruses: structural correlates of antibody neutralization of HIV-1, SARS-CoV-2, and Zika. Viruses. 2021;13(10).

6. Akkaya M, Kwak K, Pierce SK. B cell memory: building two walls of protection against pathogens. Nat Rev Immunol. 2020;20(4):229-238.

7. Altmann DM. Adaptive immunity to SARS-CoV-2. Oxf Open Immunol. 2020;1(1):iqaa003.

8. Amanna IJ, Carlson NE, Slifka MK. Duration of humoral immunity to common viral and vaccine antigens. N Engl J Med. 2007;357(19):1903-1915.

9. Antia A, Ahmed H, Handel A, et al. Heterogeneity and longevity of antibody memory to viruses and vaccines. PLoS Biol. 2018;16(8):e2006601.

10. Asokan M, Dias J, Liu C, et al. Fc-mediated effector function contributes to the in vivo antiviral effect of an HIV neutralizing antibody. Proc Natl Acad Sci U S A. 2020;117(31):18754-18763.

11. Bachmann MF, Zinkernagel RM. The influence of virus structure on antibody responses and virus serotype formation. Immunol Today. 1996;17(12):553-558.

12. Barouch DH, Whitney JB, Moldt B, et al. Therapeutic efficacy of potent neutralizing HIV-1-specific monoclonal antibodies in SHIV-infected rhesus monkeys. Nature. 2013;503(7475):224-228.

13. Batista FD, Harwood NE. The who, how and where of antigen presentation to B cells. Nat Rev Immunol. 2009;9(1):15-27.

14. Bautista JL, Cramer NT, Miller CN, et al. Single-cell transcriptional profiling of human thymic stroma uncovers novel cellular heterogeneity in the thymic medulla. Nat Commun. 2021;12(1):1096.

15. Bizebard T, Barbey-Martin C, Fleury D, et al. Structural studies on viral escape from antibody neutralization. Curr Top Microbiol Immunol. 2001;260:55-64.

16. Bjornevik K, Cortese M, Healy BC, et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. Science. 2022;375(6578):296-301.

17. Boettler T, Spangenberg HC, Neumann-Haefelin C, et al. T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. J Virol. 2005;79(12):7860-7867.

18. Bomsel M, Heyman M, Hocini H, et al. Intracellular neutralization of HIV transcytosis across tight epithelial barriers by anti-HIV envelope protein dIgA or IgM. Immunity. 1998;9(2):277-287.

19. Bottermann M, Caddy SL. Virus neutralisation by intracellular antibodies. Semin Cell Dev Biol. 2022;126:108-116.

20. Bottiger M, Gustavsson O, Svensson A. Immunity to tetanus, diphtheria and poliomyelitis in the adult population of Sweden in 1991. Int J Epidemiol. 1998;27(5):916-925.

21. Bournazos S, DiLillo DJ, Goff AJ, Glass PJ, Ravetch JV. Differential requirements for FcgammaR engagement by protective antibodies against Ebola virus. Proc Natl Acad Sci U S A. 2019;116(40):20054-20062.

22. Bournazos S, DiLillo DJ, Ravetch JV. The role of Fc-FcgammaR interactions in IgG-mediated microbial neutralization. J Exp Med. 2015;212(9):1361-1369.

23. Bournazos S, Gupta A, Ravetch JV. The role of IgG Fc receptors in antibody-dependent enhancement. Nat Rev Immunol. 2020;20(10):633-643.

24. Bournazos S, Klein F, Pietzsch J, Seaman MS, Nussenzweig MC, Ravetch JV. Broadly neutralizing anti-HIV-1 antibodies require Fc effector functions for in vivo activity. Cell. 2014;158(6):1243-1253.

25. Briney B, Inderbitzen A, Burton DR. Uniqueness, commonality and exceptional diversity in the baseline human antibody repertoire. Nature. 2018;566(7744):393-397.

26. Burioni R, Williamson RA, Sanna PP, Bloom FE, Burton DR. Recombinant human Fab to glycoprotein D neutralizes infectivity and prevents cell-to-cell transmission of herpes simplex viruses 1 and 2 in vitro. Proc Natl Acad Sci U S A. 1994;91(1):355-359.

27. Burns JW, Siadat-Pajouh M, Krishnaney AA, Greenberg HB. Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity. Science. 1996;272(5258):104-107.

28. Burton DR. Antibodies, viruses and vaccines. Nat Rev Immunol. 2002;2(9):706-713.

29. Burton DR. What are the most powerful immunogen design vaccine strategies? Reverse vaccinology 2.0 shows great promise. Cold Spring Harb Perspect Biol. 2017;9(11):a030262.

30. Burton DR, Mascola JR. Antibody responses to envelope glycoproteins in HIV-1 infection. Nat Immunol. 2015;16(6):571-576.

31. Burton DR, Topol EJ. Variant-proof vaccines—invest now for the next pandemic. Nature. 2021;590(7846):386-388.

32. Burton DR, Walker LM. Rational vaccine design in the time of COVID-19. Cell Host Microbe. 2020;27(5):695-698.

33. Burton DR, Poignard P, Stanfield RL, Wilson IA. Broadly neutralizing antibodies present new prospects to counter highly antigenically diverse viruses. Science. 2012;337(6091):183-186.

34. Cabrera R, Tu Z, Xu Y, et al. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. Hepatology. 2004;40(5):1062-1071.

35. Cacciarelli TV, Martinez OM, Gish RG, Villanueva JC, Krams SM. Immunoregulatory cytokines in chronic hepatitis C virus infection: pre- and posttreatment with interferon alfa. Hepatology. 1996;24(1):6-9.

36. Caddy SL, Vaysburd M, Wing M, et al. Intracellular neutralisation of rotavirus by VP6-specific IgG. PLoS Pathog. 2020;16(8):e1008732.

37. Cancro MP, Tomayko MM. Memory B cells and plasma cells: the differentiative continuum of humoral immunity. Immunol Rev. 2021;303(1):72-82.

38. Caskey M, Klein F, Nussenzweig MC. Broadly neutralizing anti-HIV-1 monoclonal antibodies in the clinic. Nat Med. 2019;25(4):547-553.

39. Chackerian B, Peabody DS. Factors that govern the induction of long-lived antibody responses. Viruses. 2020;12(1):74.

40. Cho A, Wrammert J. Implications of broadly neutralizing antibodies in the development of a universal influenza vaccine. Curr Opin Virol. 2016;17:110-115.

41. Corey L, Gilbert PB, Juraska M, et al.; Teams HHaHHS. Two randomized trials of neutralizing antibodies to prevent HIV-1 acquisition. N Engl J Med. 2021;384(11):1003-1014.

42. Corti D, Lanzavecchia A. Broadly neutralizing antiviral antibodies. Annu Rev Immunol. 2013;31:705-742.

43. Corti D, Misasi J, Mulangu S, et al. Protective monotherapy against lethal Ebola virus infection by a potently neutralizing antibody. Science. 2016;351(6279):1339-1342.

44. Crotty S. T follicular helper cell biology: a decade of discovery and diseases. Immunity. 2019;50(5):1132-1148.

45. Crotty S, Ahmed R. Immunological memory in humans. Semin Immunol. 2004;16:197-203.

46. Crotty S, Felgner P, Davies H, Glidewell J, Villarreal L, Ahmed R. Cutting edge: long-term B cell memory in humans after smallpox vaccination. J Immunol. 2003;171(10):4969-4973.

47. Cyster JG, Allen CDC. B cell responses: cell interaction dynamics and decisions. Cell. 2019;177(3):524-540.

48. Davenport FM, Hennessy AV, Francis T Jr. Epidemiologic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus. J Exp Med. 1953;98(6):641-656.

49. Davies DR, Padlan EA, Sheriff S. Antibody-antigen complexes. Annu Rev Biochem. 1990;59:439-473.

50. Day PM, Kines RC, Thompson CD, et al. In vivo mechanisms of vaccine-induced protection against HPV infection. Cell Host Microbe. 2010;8(3):260-270.

51. Delves PJ, Martin SJ, Burton DR, Roitt IM. Roitt's Essential Immunology. 13th ed. Wiley-Blackwell; 2017.

52. DiLillo DJ, Tan GS, Palese P, Ravetch JV. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcgammaR interactions for protection against influenza virus in vivo. Nat Med. 2014;20(2):143-151.

53. Dimmock NJ. Update on the neutralization of animal viruses. Rev Med Virol. 1995;5:165-179.

54. Dingens AS, Arenz D, Weight H, Overbaugh J, Bloom JD. An antigenic atlas of HIV-1 escape from broadly neutralizing antibodies distinguishes functional and structural epitopes. Immunity. 2019;50(2):520-532 e3.

55. Dolgin E. Pan-coronavirus vaccine pipeline takes form. Nat Rev Drug Discov. 2022;21(5):324-326.

56. Dreyfus C, Laursen NS, Kwaks T, et al. Highly conserved protective epitopes on influenza B viruses. Science. 2012;337(6100):1343-1348.

57. el-Ad B, Roth Y, Winder A, et al. The persistence of neutralizing antibodies after revaccination against smallpox. J Infect Dis. 1990;161(3):446-448.

58. Ellebedy AH. Immunizing the immune: can we overcome influenza's most formidable challenge? Vaccines. 2018;6(4):68.

59. Elsner RA, Shlomchik MJ. Germinal center and extrafollicular B cell responses in vaccination, immunity, and autoimmunity. Immunity. 2020;53(6):1136-1150.

60. Engels N, Wienands J. Memory control by the B cell antigen receptor. Immunol Rev. 2018;283(1):150-160.

61. Ercolini AM, Miller SD. Mechanisms of immunopathology in murine models of central nervous system demyelinating disease. J Immunol. 2006;176(6):3293-3298.

62. Fan W, Zhang D, Qian P, et al. Swine TRIM21 restricts FMDV infection via an intracellular neutralization mechanism. Antiviral Res. 2016;127:32-40.

63. Fujinami RS, Oldstone MB. Antiviral antibody reacting on the plasma membrane alters measles virus expression inside the cell. Nature. 1979;279(5713):529-530.

64. Fujioka H, Emancipator SN, Aikawa M, et al. Immunocytochemical colocalization of specific immunoglobulin A with sendai virus protein in infected polarized epithelium. J Exp Med. 1998;188(7):1223-1229.

65. Gerhard W. The role of the antibody response in influenza virus infection. Curr Top Microbiol Immunol. 2001;260:171-190.

66. Gourley TS, Wherry EJ, Masopust D, Ahmed R. Generation and maintenance of immunological memory. Semin Immunol. 2004;16(5):323-333.

67. Gross AJ, Hochberg D, Rand WM, Thorley-Lawson DA. EBV and systemic lupus erythematosus: a new perspective. J Immunol. 2005;174(11):6599-6607.

68. Group TI-RS. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. Pediatrics. 1998;102(3 Pt 1):531-537.

69. Gruell H, Vanshylla K, Weber T, Barnes CO, Kreer C, Klein F. Antibody-mediated neutralization of SARS-CoV-2. Immunity. 2022;55(6):925-944.

70. Gunaydin G, Yu S, Graslund T, Hammarstrom L, Marcotte H. Fusion of the mouse IgG1 Fc domain to the VHH fragment (ARP1) enhances protection in a mouse model of rotavirus. Sci Rep. 2016;6:30171.

71. Halstead SB. Immune enhancement of viral infection. Prog Allergy. 1982;31:301-364.

72. Hammarlund E, Lewis MW, Hansen SG, et al. Duration of antiviral immunity after smallpox vaccination. Nat Med. 2003;9(9):1131-1137.

72a. Hangartner L, Beauparlant D, Rakasz E, et al. Effector function does not contribute to protection from virus challenge by a highly potent HIV broadly neutralizing antibody in nonhuman primates. *Sci Transl Med*. 2021;13:eabe3349.

73. Hawkes RA, Lafferty KJ. The enchancement of virus infectivity by antibody. Virology. 1967;33(2):250-261.

74. Hawley L, Clarke B, Ziegler RJ. BRS Microbiology and Immunology. 6th ed. Lippincott Williams & Wilkins; 2013.

75. Haynes BF, Mascola JR. The quest for an antibody-based HIV vaccine. Immunol Rev. 2017;275(1):5-10.

76. Haynes LM, Vanderlugt CL, Dal Canto MC, Melvold RW, Miller SD. CD8(+) T cells from Theiler's virus-resistant BALB/cByJ mice downregulate pathogenic virus-specific CD4(+) T cells. J Neuroimmunol. 2000;106(1-2):43-52.

77. He XS, Greenberg HB. CD8+ T-cell response against hepatitis C virus. Viral Immunol. 2002;15(1):121-131.

78. Henchal EA, Henchal LS, Schlesinger JJ. Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. J Gen Virol. 1988;69(Pt 8):2101-2107.

79. Hessell AJ, Hangartner L, Hunter M, et al. Fc receptor but not complement binding is important in antibody protection against HIV. Nature. 2007;449(7158):101-104.

80. Hessell AJ, Rakasz EG, Tehrani DM, et al. Broadly neutralizing monoclonal antibodies 2F5 and 4E10 directed against the human immunodeficiency virus type 1 gp41 membrane-proximal external region protect against mucosal challenge by simian-human immunodeficiency virus SHIVBa-L. J Virol. 2010;84(3):1302-1313.

81. Hessell AJ, Poignard P, Hunter M, et al. Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. Nat Med. 2009;15(8):951-954.

82. Hessell AJ, Rakasz EG, Poignard P, et al. Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers. PLoS Pathog. 2009;5(5):e1000433.

83. Hewat E, Blaas D. Structural studies on antibody interacting with viruses. Curr Top Microbiol Immunol. 2001;260:29-44.

84. Hooks JJ, Burns W, Hayashi K, Geis S, Notkins AL. Viral spread in the presence of neutralizing antibody: mechanisms of persistence in foamy virus infection. Infect Immun. 1976;14(5):1172-1178.

85. Howcroft TK, Strebel K, Martin MA, Singer DS. Repression of MHC class I gene promoter activity by two-exon Tat of HIV. Science. 1993;260(5112):1320-1322.

86. Hu J, Robinson JL. Treatment of respiratory syncytial virus with palivizumab: a systematic review. World J Pediatr. 2010;6(4):296-300.

87. Huang C. Germinal center reaction. Adv Exp Med Biol. 2020;1254:47-53.

88. Ise W, Kurosaki T. Regulation of plasma cell differentiation. Adv Exp Med Biol. 2020;1254:63-74.

89. Ise W, Kurosaki T. Plasma cell generation during T-cell-dependent immune responses. Int Immunol. 2021;33(12):797-801.

90. Iversen PL, Kane CD, Zeng X, et al. Recent successes in therapeutics for Ebola virus disease: no time for complacency. Lancet Infect Dis. 2020;20(9):e231-e237.

91. James JA, Kaufman KM, Farris AD, Taylor-Albert E, Lehman TJ, Harley JB. An increased prevalence of Epstein-Barr virus infection in young patients suggests a possible etiology for systemic lupus erythematosus. J Clin Invest. 1997;100(12):3019-3026.

92. Jin J, Liss NM, Chen DH, et al. Neutralizing monoclonal antibodies block chikungunya virus entry and release by targeting an epitope critical to viral pathogenesis. Cell Rep. 2015;13(11):2553-2564.

93. Kato Y, Abbott RK, Freeman BL, et al. Multifaceted effects of antigen valency on B cell response composition and differentiation in vivo. Immunity. 2020;53(3):548-563 e8.

94. Kato H, Kato R, Fujihashi K, McGhee JR. Role of mucosal antibodies in viral infections. Curr Top Microbiol Immunol. 2001;260:201-228.

95. Keeler SP, Fox JM. Requirement of Fc-Fc gamma receptor interaction for antibody-based protection against emerging virus infections. Viruses. 2021;13(6):1037.

96. Kim CW, Chang KM. Hepatitis C virus: virology and life cycle. Clin Mol Hepatol. 2013;19(1):17-25.

97. Klasse PJ. Neutralization of virus infectivity by antibodies: old problems in new perspectives. Adv Biol. 2014;2014:157895.

98. Klasse PJ, Burton DR. Antibodies to West Nile virus: a double-edged sword. Cell Host Microbe. 2007;1:87-89.

99. Klasse PJ, Moore JP. Reappraising the value of HIV-1 vaccine correlates of protection analyses. J Virol. 2022;96(8):e0003422.

100. Klasse PJ, Sattentau QJ. Occupancy and mechanism in antibody-mediated neutralization of animal viruses. J Gen Virol. 2002;83(Pt 9):2091-2108.

101. Kringelum JV, Nielsen M, Padkjaer SB, Lund O. Structural analysis of B-cell epitopes in antibody:protein complexes. Mol Immunol. 2013;53(1-2):24-34.

102. Kurosaki T, Kometani K, Ise W. Memory B cells. Nat Rev Immunol. 2015;15(3):149-159.

103. Kwak K, Akkaya M, Pierce SK. B cell signaling in context. Nat Immunol. 2019;20(8):963-969.

104. Kwong PD, Mascola JR. HIV-1 vaccines based on antibody identification, B cell ontogeny, and epitope structure. Immunity. 2018;48(5):855-871.

105. Laidlaw BJ, Cyster JG. Transcriptional regulation of memory B cell differentiation. Nat Rev Immunol. 2021;21(4):209-220.

106. Lam KP, Kuhn R, Rajewsky K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell. 1997;90(6):1073-1083.

107. Lam JH, Smith FL, Baumgarth N. B cell activation and response regulation during viral infections. Viral Immunol. 2020;33(4):294-306.

108. Lanz TV, Brewer RC, Ho PP, et al. Clonally expanded B cells in multiple sclerosis bind EBV EBNA1 and GlialCAM. Nature. 2022;603(7900):321-327.

109. Lau AW, Brink R. Selection in the germinal center. Curr Opin Immunol. 2020;63:29-34.

110. Law M, Sanna PP, Burton DR. Viral subversion of humoral immune responses. In: Lachmann PJ, Oldstone MB, eds. Microbial Subversion of Immunity: Current Topics. Caister Academic Press; 2006:177-210.

111. Le Roy E, Muhlethaler-Mottet A, Davrinche C, Mach B, Davignon JL. Escape of human cytomegalovirus from HLA-DR-restricted CD4(+) T-cell response is mediated by repression of gamma interferon-induced class II transactivator expression. J Virol. 1999;73(8):6582-6589.

112. Lenzi M, Ballardini G, Fusconi M, et al. Type 2 autoimmune hepatitis and hepatitis C virus infection. Lancet. 1990;335(8684):258-259.

113. Levine B, Hardwick JM, Trapp BD, Crawford TO, Bollinger RC, Griffin DE. Antibody-mediated clearance of alphavirus infection from neurons. Science. 1991;254(5033):856-860.

114. Li H, Zony C, Chen P, Chen BK. Reduced potency and incomplete neutralization of broadly neutralizing antibodies against cell-to-cell transmission of HIV-1 with transmitted founder Envs. J Virol. 2017;91(9):e02425-16.

115. Lightman SM, Utley A, Lee KP. Survival of long-lived plasma cells (LLPC): piecing together the puzzle. Front Immunol. 2019;10:965.

116. Lilley BN, Ploegh HL. Viral modulation of antigen presentation: manipulation of cellular targets in the ER and beyond. Immunol Rev. 2005;207:126-144.

117. Lu LL, Suscovich TJ, Fortune SM, Alter G. Beyond binding: antibody effector functions in infectious diseases. Nat Rev Immunol. 2018;18(1):46-61.

118. MacCallum RM, Martin AC, Thornton JM. Antibody-antigen interactions: contact analysis and binding site topography. J Mol Biol. 1996;262(5):732-745.

119. Manns MP, Johnson EF, Griffin KJ, Tan EM, Sullivan KF. Major antigen of liver kidney microsomal autoantibodies in idiopathic autoimmune hepatitis is cytochrome P450db1. J Clin Invest. 1989;83(3):1066-1072.

120. Manz RA, Thiel A, Radbruch A. Lifetime of plasma cells in the bone marrow. Nature. 1997;388(6638):133-134.

121. Marasco WA, Sui J. The growth and potential of human antiviral monoclonal antibody therapeutics. Nat Biotechnol. 2007;25(12):1421-1434.

122. Marceau G, Lapierre P, Beland K, Soudeyns H, Alvarez F. LKM1 autoantibodies in chronic hepatitis C infection: a case of molecular mimicry? Hepatology. 2005;42(3):675-682.

123. Maruyama M, Lam KP, Rajewsky K. Memory B-cell persistence is independent of persisting immunizing antigen. Nature. 2000;407(6804):636-642.

124. Mazanec MB, Coudret CL, Fletcher DR. Intracellular neutralization of influenza virus by immunoglobulin A anti-hemagglutinin monoclonal antibodies. J Virol. 1995;69(2):1339-1343.

125. Mazanec MB, Lamm ME, Lyn D, Portner A, Nedrud JG. Comparison of IgA versus IgG monoclonal antibodies for passive immunization of the murine respiratory tract. Virus Res. 1992;23(1-2):1-12.

126. McCarthy KR, Von Holle TA, Sutherland LL, et al. Differential immune imprinting by influenza virus vaccination and infection in nonhuman primates. Proc Natl Acad Sci U S A. 2021;118(23):e2026752118.

127. McCoy LE, van Gils MJ, Ozorowski G, et al. Holes in the glycan shield of the native HIV envelope are a target of trimer-elicited neutralizing antibodies. Cell Rep. 2016;16(9): 2327-2338.

128. McCullough KC, Parkinson D, Crowther JR. Opsonization-enhanced phagocytosis of foot-and-mouth disease virus. Immunology. 1988;65(2):187-191.

129. Merkler D, Horvath E, Bruck W, Zinkernagel RM, Del la Torre JC, Pinschewer DD. “Viral deja vu” elicits organ-specific immune disease independent of reactivity to self. J Clin Invest. 2006;116(5):1254-1263.

130. Michelson DA, Hase K, Kaisho T, Benoist C, Mathis D. Thymic epithelial cells co-opt lineage-defining transcription factors to eliminate autoreactive T cells. Cell. 2022;185(14):2542-2558.e18.

131. Miller JD, Masopust D, Wherry EJ, Kaech S, Silvestri G, Ahmed R. Differentiation of CD8 T cells in response to acute and chronic viral infections: implications for HIV vaccine development. Curr Drug Targets Infect Disord. 2005;5(2):121-129.

132. Miller DM, Rahill BM, Boss JM, et al. Human cytomegalovirus inhibits major histocompatibility complex class II expression by disruption of the Jak/Stat pathway. J Exp Med. 1998;187(5):675-683.

133. Murin CD, Wilson IA, Ward AB. Antibody responses to viral infections: a structural perspective across three different enveloped viruses. Nat Microbiol. 2019;4(5):734-747.

134. Murphy KM, Weaver C. Janeway’s Immunobiology. 9th ed. W. W. Norton & Company; 2016.

135. Ngono AE, Shresta S. Immune response to dengue and Zika. Annu Rev Immunol. 2018;36:279-308.

136. Nishimura Y, Martin MA. Of mice, macaques, and men: broadly neutralizing antibody immunotherapy for HIV-1. Cell Host Microbe. 2017;22(2):207-216.

137. Ogra PL, Faden H, Welliver RC. Vaccination strategies for mucosal immune responses. Clin Microbiol Rev. 2001;14(2):430-445.

138. Oldstone MB. Molecular mimicry and autoimmune disease. Cell. 1987;50(6):819-820.

139. Ormandy LA, Hillemann T, Wedemeyer H, Manns MP, Greten TF, Korangy F. Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. Cancer Res. 2005;65(6):2457-2464.

140. Padlan EA. Anatomy of the antibody molecule. Mol Immunol. 1994;31(3):169-217.

141. Panoutsakopoulou V, Sanchirico ME, Huster KM, et al. Analysis of the relationship between viral infection and autoimmune disease. Immunity. 2001;15(1):137-147.

142. Pantaleo G, Demarest JF, Vaccarezza M, et al. Effect of anti-V3 antibodies on cell-free and cell-to-cell human immunodeficiency virus transmission. Eur J Immunol. 1995;25(1):226-231.

143. Parren PW, Burton DR. The antiviral activity of antibodies in vitro and in vivo. Adv Immunol. 2001;77:195-262.

144. Parren PW, Geisbert TW, Maruyama T, Jahrling PB, Burton DR. Pre- and postexposure prophylaxis of Ebola virus infection in an animal model by passive transfer of a neutralizing human antibody. J Virol. 2002;76(12):6408-6412.

145. Parsons MS, Lee WS, Kristensen AB, et al. Fc-dependent functions are redundant to efficacy of anti-HIV antibody PGT121 in macaques. J Clin Invest. 2019;129(1):182-191.

146. Pascal KE, Dudgeon D, Trefry JC, et al. Development of Clinical-stage human monoclonal antibodies that treat advanced Ebola virus disease in nonhuman primates. J Infect Dis. 2018;218(suppl_5):S612-S626.

147. Paul WE. Fundamental Immunology. 7th ed. Lippincott Williams & Wilkins; 2012.

148. Pegu A, Borate B, Huang Y, et al. A meta-analysis of passive immunization studies shows that serum-neutralizing antibody titer associates with protection against SHIV challenge. Cell Host Microbe. 2019;26(3):336-346 e3.

149. Phillips AJ, Tomasec P, Wang EC, Wilkinson GW, Borysiewicz LK. Human cytomegalovirus infection downregulates expression of the cellular aminopeptidases CD10 and CD13. Virology. 1998;250(2):350-358.

150. Plotkin SA, Orenstein WA. Vaccines. W.B. Saunders Co.; 1999:1230.

151. Prince GA, Horswood RL, Chanock RM. Quantitative aspects of passive immunity to respiratory syncytial virus infection in infant cotton rats. J Virol. 1985;55(3):517-520.

152. Punt J, Stranford S, Jones P, Owen J. Kuby Immunology. 8th ed. W. H. Freeman; 2018.

153. Qiu X, Wong G, Audet J, et al. Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. Nature. 2014;514(7520):47-53.

154. Raghunandan R, Higgins D, Hosken N. RSV neutralization assays—use in immune response assessment. Vaccine. 2021;39(33):4591-4597.

155. Ramilo O, Lagos R, Saez-Llorens X, et al. Motavizumab treatment of infants hospitalized with respiratory syncytial virus infection does not decrease viral load or severity of illness. Pediatr Infect Dis J. 2014;33(7):703-709.

156. Rappuoli R, Bottomley MJ, D'Oro U, Finco O, De Gregorio E. Reverse vaccinology 2.0: human immunology instructs vaccine antigen design. J Exp Med. 2016;213(4):469-481.

157. Ratcliffe MJH. Encyclopedia of Immunobiology. 1st ed. Academic Press; 2016.

158. Reh L, Magnus C, Schanz M, et al. Capacity of broadly neutralizing antibodies to inhibit HIV-1 cell-cell transmission is strain- and epitope-dependent. PLoS Pathog. 2015;11(7):e1004966.

159. Ren X, Sodroski J, Yang X. An unrelated monoclonal antibody neutralizes human immunodeficiency virus type 1 by binding to an artificial epitope engineered in a functionally neutral region of the viral envelope glycoproteins. J Virol. 2005;79(9):5616-5624.

160. Richman DD, Wrin T, Little SJ, Petropoulos CJ. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc Natl Acad Sci U S A. 2003;100(7):4144-4149.

161. Robinson MJ, Webster RH, Tarlinton DM. How intrinsic and extrinsic regulators of plasma cell survival might intersect for durable humoral immunity. Immunol Rev. 2020;296(1):87-103.

162. Roco JA, Mesin L, Binder SC, et al. Class-switch recombination occurs infrequently in germinal centers. Immunity. 2019;51(2):337-350 e7.

163. Roltgen K, Nielsen SCA, Silva O, et al. Immune imprinting, breadth of variant recognition, and germinal center response in human SARS-CoV-2 infection and vaccination. Cell. 2022;185(6):1025-1040 e14.

164. Saphire EO, Schendel SL, Gunn BM, Milligan JC, Alter G. Antibody-mediated protection against Ebola virus. Nat Immunol. 2018;19(11):1169-1178.

165. Schafer A, Muecksch F, Lorenzi JCC, et al. Antibody potency, effector function, and combinations in protection and therapy for SARS-CoV-2 infection in vivo. J Exp Med. 2021;218(3).

166. Schiffner T, Sattentau QJ, Duncan CJ. Cell-to-cell spread of HIV-1 and evasion of neutralizing antibodies. Vaccine. 2013;31(49):5789-5797.

167. Schlender J, Hornung V, Finke S, et al. Inhibition of toll-like receptor 7- and 9-mediated alpha/beta interferon production in human plasmacytoid dendritic cells by respiratory syncytial virus and measles virus. J Virol. 2005;79(9):5507-5515.

168. Schlesinger JJ, Chapman S. Neutralizing F(ab')2 fragments of protective monoclonal antibodies to yellow fever virus (YF) envelope protein fail to protect mice against lethal YF encephalitis. J Gen Virol. 1995;76(Pt 1):217-220.

169. Seifert M, Kuppers R. Human memory B cells. Leukemia. 2016;30(12):2283-2292.

170. Servet C, Zitvogel L, Hosmalin A. Dendritic cells in innate immune responses against HIV. Curr Mol Med. 2002;2(8):739-756.

171. Sette A, Moutaftsi M, Moyron-Quiroz J, et al. Selective CD4+ T cell help for antibody responses to a large viral pathogen: deterministic linkage of specificities. Immunity. 2008;28(6):847-858.

172. Simmons RA, Willberg CB, Paul K. Immune Evasion by Viruses. John Wiley & Sons, Ltd; 2013. doi:10.1002/9780470015902.a0024790

173. Slifka MK. Immunological memory to viral infection. Curr Opin Immunol. 2004;16(4):443-450.

174. Slifka MK, Ahmed R. Long-term humoral immunity against viruses: revisiting the issue of plasma cell longevity. Trends Microbiol. 1996;4(10):394-400.

175. Slifka MK, Antia R, Whitmire JK, Ahmed R. Humoral immunity due to long-lived plasma cells. Immunity. 1998;8(3):363-372.

176. Smith TJ. Antibody interactions with rhinovirus: lessons for mechanisms of neutralization and the role of immunity in viral evolution. Curr Top Microbiol Immunol. 2001;260:1-28.

177. Smith TJ. Structural studies on antibody-virus complexes. Adv Protein Chem. 2003;64:409-453.

178. Sobue S, Nomura T, Ishikawa T, et al. Th1/Th2 cytokine profiles and their relationship to clinical features in patients with chronic hepatitis C virus infection. J Gastroenterol. 2001;36(8):544-551.

179. Sompayrac LM. *How the Immune System Works*. 6th ed. Wiley-Blackwell; 2019.

180. Spear GT, Hart M, Olinger GG, Hashemi FB, Saifuddin M. The role of the complement system in virus infections. Curr Top Microbiol Immunol. 2001;260:229-245.

181. Sterling RK, Bralow S. Extrahepatic manifestations of hepatitis C virus. Curr Gastroenterol Rep. 2006;8(1):53-59.

182. Sugimoto K, Ikeda F, Stadanlick J, Nunes FA, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. Hepatology. 2003;38(6):1437-1448.

183. Sullivan NJ. Antibody-mediated enhancement of viral disease. Curr Top Microbiol Immunol. 2001;260:145-169.

184. Sullivan N, Sun Y, Li J, Hofmann W, Sodroski J. Replicative function and neutralization sensitivity of envelope glycoproteins from primary and T-cell line-passaged human immunodeficiency virus type 1 isolates. J Virol. 1995;69(7):4413-4422.

185. Sundberg EJ, Mariuzza RA. Molecular recognition in antibody-antigen complexes. Adv Protein Chem. 2002;61:119-160.

186. Suphaphiphat K, Tolazzi M, Hua S, et al. Broadly neutralizing antibodies potently inhibit cell-to-cell transmission of semen leukocyte-derived SHIV162P3. EBioMedicine. 2020;57:102842.

187. Sutton TC, Lamirande EW, Bock KW, et al. In vitro neutralization is not predictive of prophylactic efficacy of broadly neutralizing monoclonal antibodies CR6261 and CR9114 against lethal H2 influenza virus challenge in mice. J Virol. 2017;91(24):e01603-e01617.

188. Suvas S, Azkur AK, Kim BS, Kumaraguru U, Rouse BT. CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. J Immunol. 2004;172(7):4123-4132.

189. Suvas S, Kumaraguru U, Pack CD, Lee S, Rouse BT. CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. J Exp Med. 2003;198(6):889-901.

190. Tarlinton D. Do plasma cells contribute to the determination of their lifespan? Immunol Cell Biol. 2020;98(6):449-455.

191. Tellier J, Nutt SL. Plasma cells: the programming of an antibody-secreting machine. Eur J Immunol. 2019;49(1):30-37.

192. Topham DJ, Tripp RA, Doherty PC. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. J Immunol. 1997;159(11):5197-5200.

193. Tumpey TM, Basler CF, Aguilar PV, et al. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science. 2005;310(5745):77-80.

194. Vaidehi Narayanan H, Hoffmann A. From antibody repertoires to cell-cell interactions to molecular networks: bridging scales in the germinal center. Front Immunol. 2022;13:898078.

195. Vatti A, Monsalve DM, Pacheco Y, Chang C, Anaya JM, Gershwin ME. Original antigenic sin: a comprehensive review. J Autoimmun. 2017;83:12-21.

196. Victora GD, Nussenzweig MC. Germinal centers. Annu Rev Immunol. 2022;40:413-442.

197. Walker LM, Burton DR. Passive immunotherapy of viral infections: 'super-antibodies' enter the fray. Nat Rev Immunol. 2018;18(5):297-308.

198. Walzl G, Tafuro S, Moss P, Openshaw PJ, Hussell T. Influenza virus lung infection protects from respiratory syncytial virus-induced immunopathology. J Exp Med. 2000;192(9):1317-1326.

199. Wang P, Gajjar MR, Yu J, et al. Quantifying the contribution of Fc-mediated effector functions to the antiviral activity of anti-HIV-1 IgG1 antibodies in vivo. Proc Natl Acad Sci U S A. 2020;117(30):18002-18009.

200. Watkinson RE, McEwan WA, Tam JC, Vaysburd M, James LC. TRIM21 Promotes cGAS and RIG-I sensing of viral genomes during infection by antibody-opsonized virus. PLoS Pathog. 2015;11(10):e1005253.

201. Wei X, Decker JM, Wang S, et al. Antibody neutralization and escape by HIV-1. Nature. 2003;422(6929):307-312.

202. Weisel F, Shlomchik M. Memory B cells of mice and humans. Annu Rev Immunol. 2017;35:255-284.

203. Welsh RM, Selin LK, Szomolanyi-Tsuda E. Immunological memory to viral infections. Annu Rev Immunol. 2004;22:711-743.

204. Wessel AW, Kose N, Bombardi RG, et al. Antibodies targeting epitopes on the cell-surface form of NS1 protect against Zika virus infection during pregnancy. Nat Commun. 2020;11(1):5278.

205. Williamson LE, Reeder KM, Bailey K, et al. Therapeutic alphavirus cross-reactive E1 human antibodies inhibit viral egress. Cell. 2021;184(17):4430-4446 e22.

206. Wilson IA, Stanfield RL. Antibody-antigen interactions: new structures and new conformational changes. Curr Opin Struct Biol. 1994;4(6):857-867.

207. Winkler ES, Gilchuk P, Yu J, et al. Human neutralizing antibodies against SARS-CoV-2 require intact Fc effector functions for optimal therapeutic protection. Cell. 2021;184(7):1804-1820 e16.

208. Wohlbold TJ, Chromikova V, Tan GS, et al. Hemagglutinin stalk- and neuraminidase-specific monoclonal antibodies protect against lethal H10N8 influenza virus infection in mice. J Virol. 2016;90(2):851-861.

209. Wright KE, Buchmeier MJ. Antiviral antibodies attenuate T-cell-mediated immunopathology following acute lymphocytic choriomeningitis virus infection. J Virol. 1991;65(6):3001-3006.

210. Wyatt R, Sodroski J. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science. 1998;280(5371):1884-1888.

211. Yamamoto AM, Cresteil D, Homberg JC, Alvarez F. Characterization of anti-liver-kidney microsome antibody (anti-LKM1) from hepatitis C virus-positive and -negative sera. Gastroenterology. 1993;104(6):1762-1767.

212. Yewdell JW, Santos JJS. Original antigenic sin: how original? How sinful? Cold Spring Harbor Perspect Med. 2021;11(5):a038786.

213. Yoo JS, Sasaki M, Cho SX, et al. SARS-CoV-2 inhibits induction of the MHC class I pathway by targeting the STAT1-IRF1-NLRC5 axis. Nat Commun. 2021;12(1):6602.

214. Yu X, Tsibane T, McGraw PA, et al. Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. Nature. 2008;455(7212):532-536.

215. Yuan M, Huang D, Lee CD, et al. Structural and functional ramifications of antigenic drift in recent SARS-CoV-2 variants. Science. 2021;373(6556):818-823.

216. Yuan M, Liu H, Wu NC, et al. Structural basis of a shared antibody response to SARS-CoV-2. Science. 2020(6507):1119-1123.

217. Zandman-Goddard G, Shoenfeld Y. Infections and SLE. Autoimmunity. 2005;38(7):473-485.

218. Zhang Y, Chen Y, Li Y, et al. The ORF8 protein of SARS-CoV-2 mediates immune evasion through down-regulating MHC-Iota. Proc Natl Acad Sci U S A. 2021;118(23):e2024202118.

219. Zhang S, Loy T, Ng TS, et al. A human antibody neutralizes different flaviviruses by using different mechanisms. Cell Rep. 2020;31(4):107584.

220. Zinkernagel RM. Maternal antibodies, childhood infections, and autoimmune diseases. N Engl J Med. 2001;345(18):1331-1335.

CHAPTER 11

Tumor Virology

Daniel DiMaio • James M. Pipas

Brief introduction to cancer and cell transformation

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Cell transformation as a model of cancer

History of tumor virology

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Cellular targets of tumor viruses

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Insertional activation of cellular protooncogenes by nonacute transforming retroviruses

Mechanisms of DNA virus oncogene action

DNA virus oncoproteins that activate mitogenic signaling pathways

DNA virus oncoproteins that inactivate tumor suppressor pathways

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Human tumor virology

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Human tumor virus action

Human tumor viruses and HIV

Human papillomaviruses

Merkel cell polyomavirus

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Kaposi sarcoma–associated herpesvirus

Hepatitis B virus

Hepatitis C virus

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Human immunodeficiency virus

Human endogenous retroviruses

Future perspectives

Acknowledgments

Studies of tumor viruses have provided fundamental insights into carcinogenesis, identified the causes of some human cancers, and led to novel strategies to prevent and treat these cancers. Some viruses cause cancer by affecting the activity of crucial regulatory nodes that normally control cell proliferation and survival, whereas others indirectly enhance tumor formation by influencing cellular mutagenesis, inflammation, and

immunity. Because of their intimate association with central cellular machinery, tumor viruses are important tools for understanding all human cancers, not just those induced by viruses. For example, studies of tumor viruses led to the discovery of cellular oncogenes and tumor suppressor proteins and to the elucidation of many important aspects of mitogenic signal transduction, cell cycle control, and carcinogenesis. Indeed, the concept that cancer is a genetic disease emerged largely from tumor virology. In addition, viruses can induce specific types of cancer in humans, and cancer risk is reduced by vaccines and antiviral agents that prevent or treat infection by some of these viruses.[247] This chapter reviews some of these remarkable successes and discusses some gaps in our knowledge.

Brief Introduction to Cancer and Cell Transformation

Cancer as a Disease

Cancer, a collection of diseases characterized by inappropriate cell survival and proliferation, is responsible for ~9.5 million deaths annually worldwide. Agents that cause cancer include ionizing radiation and ultraviolet light, chemical carcinogens, genetic predisposition, and specific infectious agents, including some viruses. Cancer can be prevented by exposure reduction (eg, smoking cessation for lung cancer, avoidance of excessive ultraviolet light for skin cancer), screening and early treatment programs (eg, colonoscopies for colon cancer and Pap smear screening for cervical cancer), and vaccination against some virus-induced cancers.

Each cancer is characterized by a unique landscape of mutations, chromosomal rearrangements, copy number variants, and epigenetic and gene expression profiles. Tumorigenesis is largely the consequence of mutations in genes that regulate key cellular processes such as proliferation, cell death, metabolism, and response to stress. Mutations that directly contribute to tumor growth and survival are known as driver mutations. Because many cancers are genetically unstable, tumor cells also carry passenger mutations with no known role in tumorigenesis. Distinguishing driver and passenger mutations and determining their consequences is a major focus of cancer research.

Two major classes of driver genes are oncogenes and tumor suppressor genes. These genes often encode proteins or noncoding RNAs (ncRNAs) that function as key regulators of cell proliferation, death, or genomic integrity. Oncogenes stimulate tumorigenesis and are typically activated or overexpressed in cancer, whereas tumor suppressors inhibit tumorigenesis and are inactivated or deleted in cancer. Although in rare cases these mutations are inherited, the most frequent cause of oncogene activation or tumor suppressor inactivation are mutations occurring after fertilization (known as somatic mutations). Thus, many chemical and physical carcinogens are mutagens. Some tumor viruses encode proteins that directly activate cellular oncogenes, inactivate tumor suppressors, or regulate the pathways controlled by oncogenes and tumor suppressor genes, whereas others act more indirectly, for example by affecting genome integrity and mutagenesis, resulting in progressive loss of cell growth control. The elucidation of the roles of oncogenes, tumor suppressor genes, and mutations in cancer is a major accomplishment of biomedical research, with direct impact on cancer prevention and therapy. Studies of tumor viruses have played an outsized role in these discoveries.

Cell Transformation as a Model of Cancer

Most studies of tumor viruses are performed in cultured cells. Many noncancer cells growing in culture have strict requirements for survival and proliferation. For example, adherent cells often cease proliferation when they reach confluence on a surface (ie, display contact inhibition), require attachment to a solid surface for growth, depend on serum in the culture medium, and display a finite lifespan. In contrast, cells isolated from tumors often display altered properties such as escape from contact inhibition, ability to form multicellular foci on a surface or colonies in semisolid medium, reduced serum requirements, and unlimited growth potential. Most strikingly, these cells often form tumors upon inoculation into animals. Cells displaying these properties are called transformed cells, although some transformed cells display only a subset of these phenotypes. Infection with some viruses that induce tumors in animals can transform cells in culture. In most cases, viral DNA persists in transformed cells, but the cells usually do not express all viral genes and do not produce infectious virus. In fact, in many cases productive infection is incompatible with transformation, because virus production can result in cell death. Therefore, in transformed (and tumor) cells, viral replication functions are often inactivated by mutation, integration of the viral genome into the cell genome, or other events. In general, maintenance of the transformed phenotype requires continuous expression of viral oncogenes.

Studies of transgenic mice expressing viral oncogenes in specific cell types in their natural tissue setting have also been valuable and mitigate some of the potential artifacts of studying cultured cells. Importantly, mouse models allow a combination of viral and host genetics, in which wild-type or mutant viral oncoproteins are expressed in different mouse strains or in gene knockout backgrounds. Among other contributions, studies of transgenic mice demonstrated that viral oncogenes can induce tumors in the correct tissue context and that the host cell genetic background can greatly influence cancer risk.

History of Tumor Virology

Discovery of Tumor Viruses and Viral Oncogenes

Early studies of tumor viruses focused on isolation of viruses from naturally occurring tumors in animals and description of the effects of these viruses in experimental animals. The first tumors shown to be transmitted by viruses were avian myeloblastosis in 1908 and avian sarcomas in 1911, caused by avian myeloblastosis virus (AMV) and Rous sarcoma virus (RSV), respectively.[99,238] Study of these and related retroviruses and their mechanisms of oncogenesis provided numerous important insights into pathways of growth regulation and carcinogenesis. Indeed, some of the most important proteins in signal transduction and human cancer including Ras and Myc were first identified as retroviral oncoproteins. Diverse taxonomic groups of viruses with DNA genomes can also cause tumors: polyomaviruses, papillomaviruses, adenoviruses, herpesviruses, hepadnaviruses, and poxviruses. Thus, the ability to induce tumors is not associated with a particular virus family, genome structure, or mode of virus replication. The types of tumors induced by viruses are also diverse and include sarcomas (tumors of mesenchymal cells), leukemias and lymphomas (tumors of hematopoietic cells), and carcinomas (tumors of epithelial cells).

The first identified viral oncogene was the *src* oncogene of RSV. Retroviruses have an RNA genome, which is reverse transcribed into double-stranded DNA that integrates into the host cell's genome. This integrated viral DNA (known as a provirus) consists of three genes—*gag*, *pol*, and *env*—that encode proteins required for virus replication (Fig. 11.1A). Infection of chick embryo fibroblasts with RSV causes cell transformation, but RSV mutants exist that lost tumorigenic and transforming activity without affecting viral replication.[89] Thus, oncogenesis and transformation are not required for RSV replication. The transformation-defective mutant genome was smaller than wild-type RSV, suggesting

that viral sequences responsible for transforming activity—an oncogene eventually named *src*—were missing from the mutant (Fig. 11.1A).[66] A key discovery was the origin of *src*. Hybridization experiments with *src*-specific DNA probes revealed the presence of homologous sequences in DNA from uninfected chickens and other species.[271] Thus, the *src* gene of RSV, designated *v-src*, was derived from a normal cell gene named *c-src*, which is present in the germline of all vertebrates. This progenitor cellular gene is referred to as a proto-oncogene and was incorporated in the recent past into the retroviral genome. DNA tumor viruses also contain viral oncogenes, but usually they are intrinsic viral genes that were not recently acquired from the cell. For many virus-induced cancers, viral oncogenes play a predominant role, but for others, viral oncogenes play little if any role.

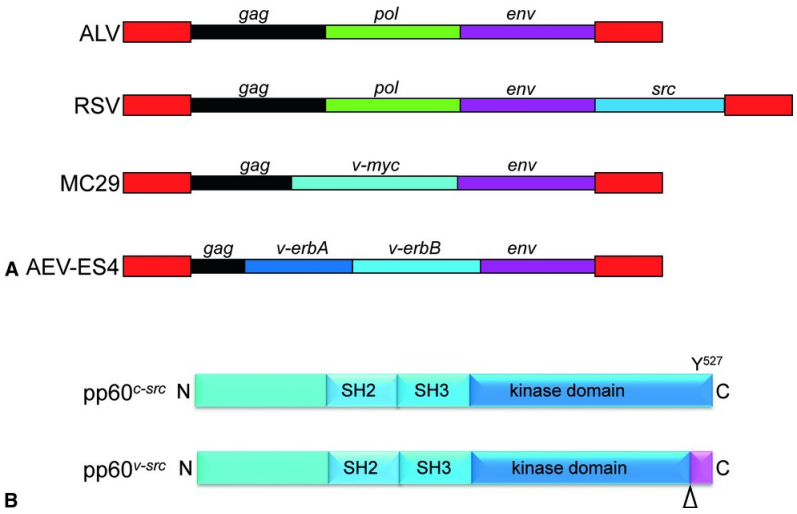

FIGURE 11.1 **Retroviral genomes and oncogenes**. **A**. Genome maps of the indicated avian retroviruses. The red segments represent the viral long terminal repeats (LTRs). The *black*, *green*, and *purple* segments represent the viral replication proteins, *gag*, *pol*, and *env*, respectively. Viral oncogenes are shown in blue. ALV (avian leukosis virus) does not contain a viral oncogene and is not an acute transforming virus. **B**. Schematic diagrams of *src* oncoproteins. $pp60^{c\text{-}src}$ is shown at the top and $pp60^{v\text{-}src}$ is shown at the *bottom*. Structural domains of both proteins are labeled, as is the C-terminal regulatory tyrosine at position 527 (Y527) in $pp60^{c\text{-}src}$. In $pp60^{v\text{-}src}$, the site of the frameshift mutation is indicated by the *triangle*, with out-of-frame sequences downstream of the mutation shown in *lavender*.

Development of New Tools that Revolutionized Biology

Intense studies of tumor viruses in the 1970s and 1980s led to the development of many important research tools and reagents, including restriction mapping, site-directed mutagenesis, gene transfer, whole genome sequencing, and recombinant DNA technology. Widespread application of these tools revolutionized genetics and molecular biology.[84,225]

These techniques were largely developed with small DNA tumor viruses because the virus particle and its genome could be isolated in pure, clonal form from infected, permissive cells for detailed analysis. The key advance for DNA analysis was the use of restriction endonucleases to study DNA isolated from tumor viruses, primarily the monkey polyomavirus, SV40. Crucially, these enzymes cleave DNA at specific, short nucleotide sequences and generate specific fragments of DNA, which can be placed in their proper order in a "restriction" map of the viral genome (Fig. 11.2).[77,79] Genetic elements such as the origin of viral DNA replication and viral transcription units could then be positioned on the map. Restriction endonucleases were also used to localize mutations[78] on the viral genome, compare viral strains, and construct specific mutations at predetermined sites in the genome for functional studies.[206] In addition, transfection techniques were developed to introduce viral DNA segments into cultured cells to determine their biological activities. Reverse transcriptase, a retroviral enzyme that catalyzes synthesis of DNA from an RNA template, was used to convert cellular RNA into DNA. Importantly, restriction endonucleases and reverse transcriptase allowed molecular cloning of cellular DNA in recombinant form, providing an unlimited supply of pure cellular genes for analysis. These techniques allowed the analysis of ever larger genomes, beginning with SV40 itself and bacteriophage ΦX174, and culminated in the complete sequencing of the genomes of many higher organisms including humans. These advances revolutionized our understanding of biology, gave birth to the biotechnology industry, and continue to transform clinical medicine.

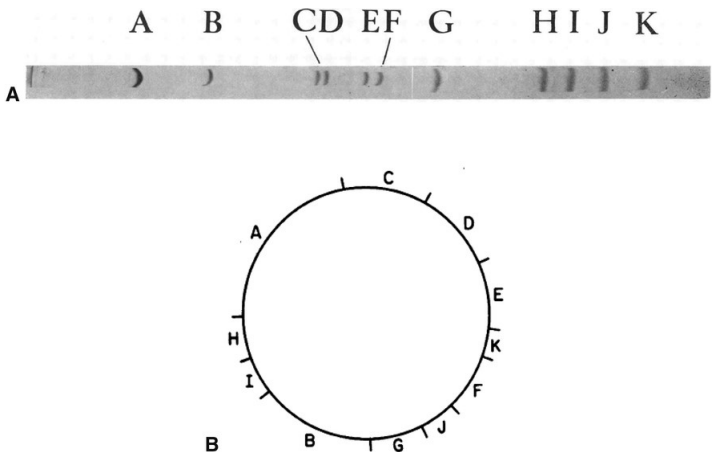

FIGURE 11.2 **Restriction endonuclease digestion generates discrete DNA fragments that can be assembled into a physical map of the viral genome**. **A**. Radiolabeled SV40 DNA was digested with restriction endonucleases from *Haemophilus influenzae* and subjected to polyacrylamide gel electrophoresis followed by autoradiography. Origin of the gel is at the left, and DNA fragments are indicated by letters. (Reprinted from Danna K, Nathans D. Specific cleavage of simian virus 40 DNA by restriction endonuclease of Hemophilus influenzae. *Proc Natl Acad Sci U S A*. 1971;68(12):2913-2917.) **B**.*H influenzae* restriction map of the circular SV40 genome. (Reprinted from Danna KJ, Nathans D. Bidirectional replication of Simian Virus 40 DNA. *Proc Natl Acad Sci U S A*. 1972;69(11):3097-3100.)

Studies of tumor viruses themselves shed light on many areas of biology including fundamental aspects of eukaryotic gene expression, DNA replication, cell signaling, protein trafficking, and cell cycle control. Transcription factors, nuclear localization and polyadenylation signals, transcriptional enhancers, and mRNA splicing, for example, were discovered by studies of small DNA tumor viruses.[84,225] The impact of these studies is highlighted by the numerous Nobel Prizes awarded to tumor virologists (Table 11.1).

TABLE 11.1 Nobel prizes awarded for studies of tumor viruses or HIV

1966	Peyton Rous—*discovery of tumor viruses*
1975	Renato Dulbecco, Howard Temin, and David Baltimore—*studies on tumor viruses; discovery of reverse transcription*
1976	Baruch Blumberg—*discovery of Hepatitis B virus*
1978	Daniel Nathans—*use of restriction enzymes to analyze SV40 DNA*
1980	Paul Berg—*construction of recombinant DNA molecules containing SV40 DNA*
1988	Gertrude Elion—*development of antiviral drugs*
1989	Michael Bishop and Harold Varmus—*discovery of the cellular origin of retroviral oncogenes*
1993	Richard Roberts and Phillip Sharp—*discovery of mRNA splicing in adenovirus*
2008	Harald zur Hausen, Luc Montagnier, and Françoise Barré-Sinoussi—*discovery of the role of human papillomavirus in cervical cancer; discovery of human immunodeficiency virus*
2020	Michael Alter, Harvey Houghton and Charles Rice—*discovery of hepatitis C virus*

Cellular Targets of Tumor Viruses

In untransformed cells, cell division is regulated by intrinsic oscillators that ensure the orderly progression of the cell division cycle and by external stimuli that dictate entry or exit from the cell cycle or modulate the activity of the cell cycle machinery. Like soluble growth factors, many viral oncoproteins stimulate DNA synthesis. Thus, the signal transduction pathways activated by growth factors are a useful framework to understand the function of viral oncogenes (Fig. 11.3).

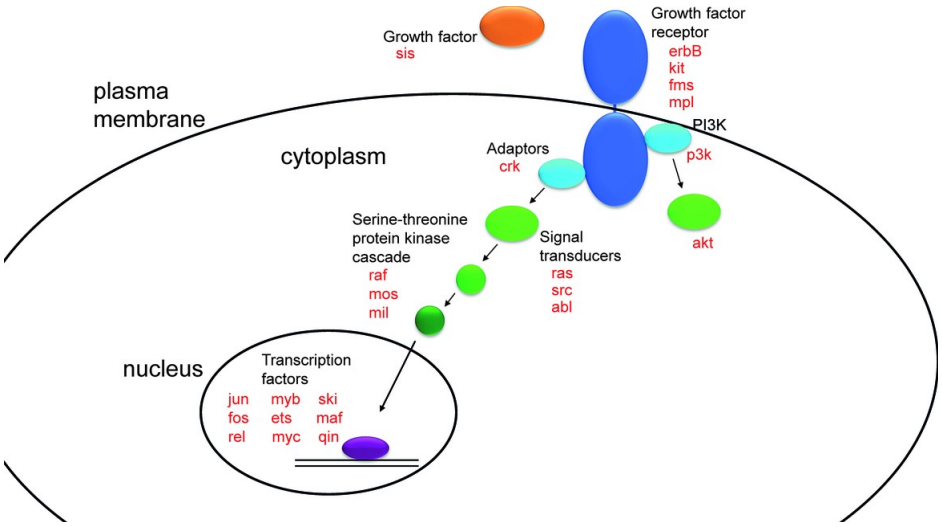

FIGURE 11.3 **Mitogenic signal transduction pathways and retroviral oncogenes**. Schematic representation of mitogenic signal transduction from the cell surface. Binding of a growth factor to a receptor tyrosine kinase initiates signal transduction pathways involving adaptor proteins, signal transducing proteins, and serine/threonine kinases, indicated by the colored ovals. This results in the phosphorylation of transcription factors to alter gene expression and execute pro-proliferative or antiapoptotic signals. Components of this pathway transduced by acute transforming retroviruses are listed in red.

Most cells grown in culture require a basal nutrient medium supplemented with serum, which contains numerous factors required for cell attachment to a surface, cell survival, and proliferation. Polypeptide growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) bind to specific cell surface receptors to initiate mitogenic signaling. Growth factor receptors are often transmembrane proteins with an extracellular ligand binding domain, a membrane-spanning domain, and an intracellular catalytic domain. For receptor tyrosine kinases (RTKs), ligand binding induces dimerization and/or reorientation of the receptors, resulting in activation of the tyrosine kinase activity of the receptors and in tyrosine phosphorylation of the receptor itself (Fig. 11.4). Receptor tyrosine phosphorylation in turn generates specific binding sites for a variety of signaling proteins and initiates a cytoplasmic signaling cascade involving a series of serine/threonine kinases and associated proteins that ultimately causes the activation of transcription factors, such as activator protein-1 (AP-1) and Myc, resulting in altered expression of various cellular genes (Fig. 11.3).[27,29,61,240]

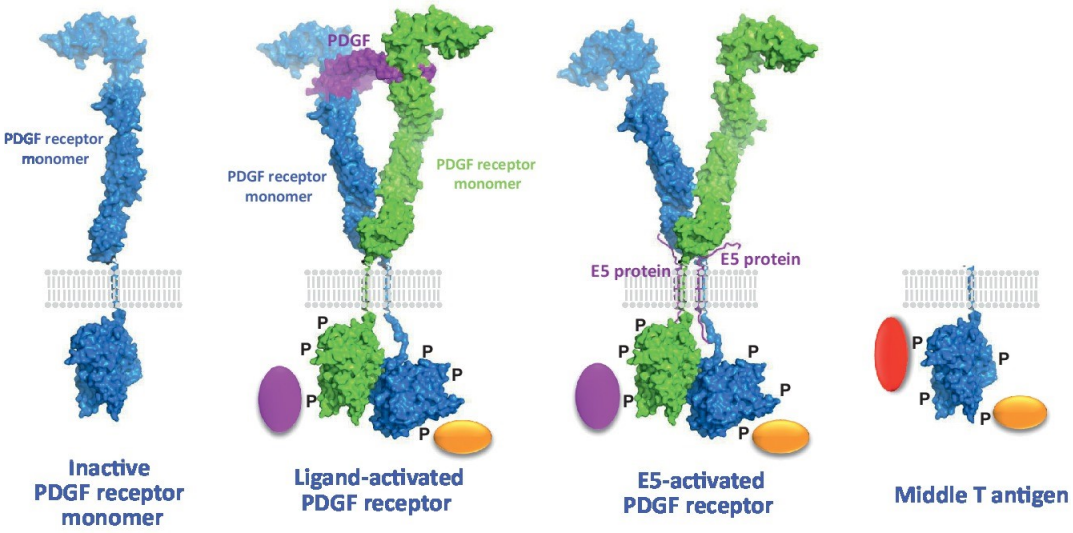

FIGURE 11.4 **DNA virus oncoproteins that mimic or activate receptor tyrosine kinases**. The left diagram shows a monomeric, inactive receptor tyrosine kinase with the cytoplasmic kinase domain at the bottom. The second diagram from left shows a dimer of two PDGF receptor molecules activated by PDGF binding. The Ps in the cytoplasmic domain represent phosphorylated tyrosines that serve as docking sites for signaling proteins, which are represented by ovals. Third diagram from left shows a dimer of two PDGF receptor molecules activated by the BPV E5 protein binding to the transmembrane domain of the receptor. The current model of the E5 protein/PDGF receptor interaction proposes two E5 dimers in the complex,[159] but only two E5 molecules are shown here for clarity. The diagram on the right shows phosphorylated murine polyomavirus middle T antigen bound to cellular signaling proteins. (Modified from DiMaio D. Viral miniproteins. *Annu Rev Microbiol*. 2014;68:21-43. Copyright © 2014 by Annual Reviews.)

The signaling cascade mobilized by growth factor treatment leads to cell cycle entry.[166] Progression to the G1 phase of the cell cycle results in the increased expression of several hundred genes that encode proteins that function in nucleotide metabolism, DNA replication, and cell cycle progression. The expression of these genes is controlled by the E2F-family of transcription factors in association with a member of the retinoblastoma family of tumor suppressors, which consists of three proteins, $p105^{Rb}$, p130, and p107 (collectively called RB). During growth arrest, E2F4/5 recruits p130 to the promoters of E2F-regulated genes and anchors a multiprotein complex that represses transcription (Fig. 11.5). Mitogenic signaling increases the expression of cyclin D, which binds and activates the cyclin-dependent kinases (cdks), cdk4 and cdk6.

Activated cdk4 and cdk6 phosphorylate p130, as well as the other RB proteins, thereby inhibiting their interaction with E2F4/5. This leads to the degradation of the repressive complex and to derepression of E2F1, E2F2, and E2F3, which then bind the promoters of E2F-regulated genes and stimulate transcription (Fig. 11.5).

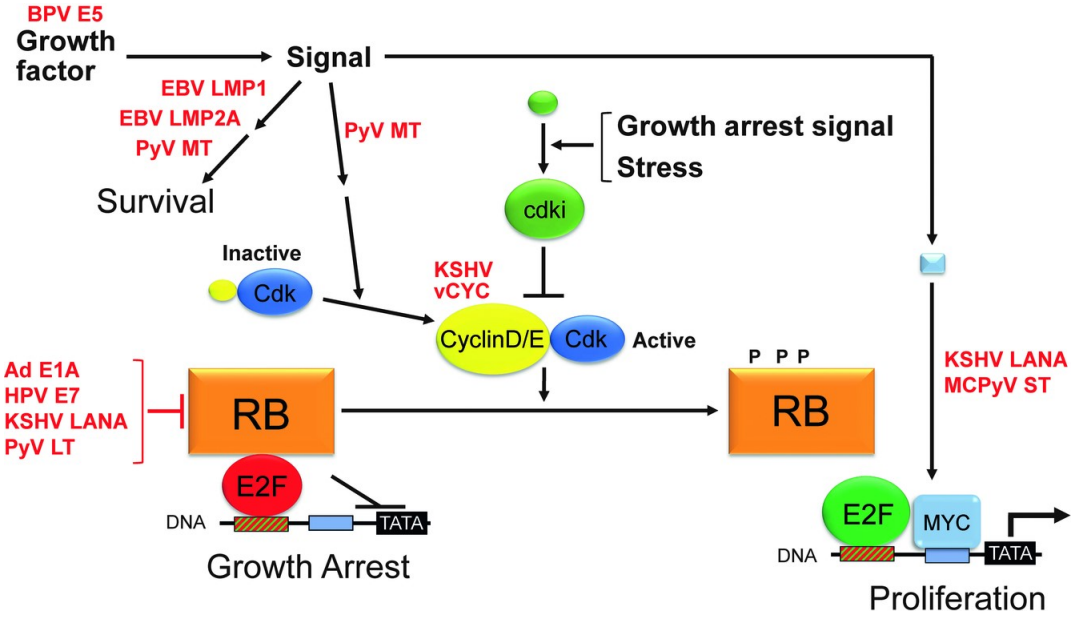

FIGURE 11.5 **Multiple DNA tumor viruses antagonize RB function**. During growth arrest, E2F-binding sites in promoters of growth-regulated genes are occupied by a repressive complex containing an E2F family member bound to an RB family member. Growth factors initiate a signaling cascade that ultimately leads to increased levels of Myc and cyclin family members. Cyclin binding to cyclin-dependent kinase (cdk) stimulates phosphorylation of RB and loss of the repressive complex. The promoters are then occupied by activating members of the E2F family and Myc, resulting in increased transcription of genes required for cell cycle progression and cell proliferation. Growth arrest or stress signals activate cdki's that inhibit cdk activity. As described in the text, multiple DNA tumor virus oncoproteins shown in red promote cell survival, stimulate RB phosphorylation, directly inhibit RB action, or induce expression of Myc family members. PyV MT, murine polyomavirus middle T antigen.

Cells often respond to stress by halting cell cycle entry even in the presence of a mitogenic signal, due to increased expression and activation of proteins that inhibit cdks (cdk inhibitors, cdki's) (Fig. 11.5). The *INK4A* family of cdki's, exemplified by p16 (*CDKN2A*), specifically inhibit cdk4/6. When p16 is active, RB proteins remain hypo-phosphorylated, and E2F-regulated genes are repressed. The WAF1 family of cdki's, exemplified by p21 (*CDKN1A*), inhibits multiple cdks and halts cell cycle progression at any stage. The expression of p21 is controlled by the tumor suppressor p53, which was discovered as cellular binding partner of SV40 large T antigen, a protein required for viral DNA replication.[177,188] p53 is a transcription factor that integrates responses to multiple stresses such as hypoxia, nutrient depletion, and DNA damage (Fig. 11.6). Under normal conditions, p53 levels are low due to a feedback loop with the ubiquitin ligase mdm2: p53 stimulates transcription of the mdm2 gene and the resulting increased mdm2 levels cause p53 ubiquitination and degradation. Stress activates kinases that phosphorylate p53 and render it unable to interact with mdm2. This results in increased p53 levels that then activate transcription of p21, leading to cell cycle arrest. Prolonged p53 activation leads to increased levels of cell death proteins such as Bax, resulting in apoptosis. Activation of mitogenic signaling or inhibition of tumor suppressor proteins such as RB and p53 can stimulate cell proliferation or enhance cell survival. Not surprisingly, most viral oncogenes activate signal transduction cascades or inhibit tumor suppressor pathways or the cell cycle machinery (Figs. 11.3, 11.5, and 11.6).[26]

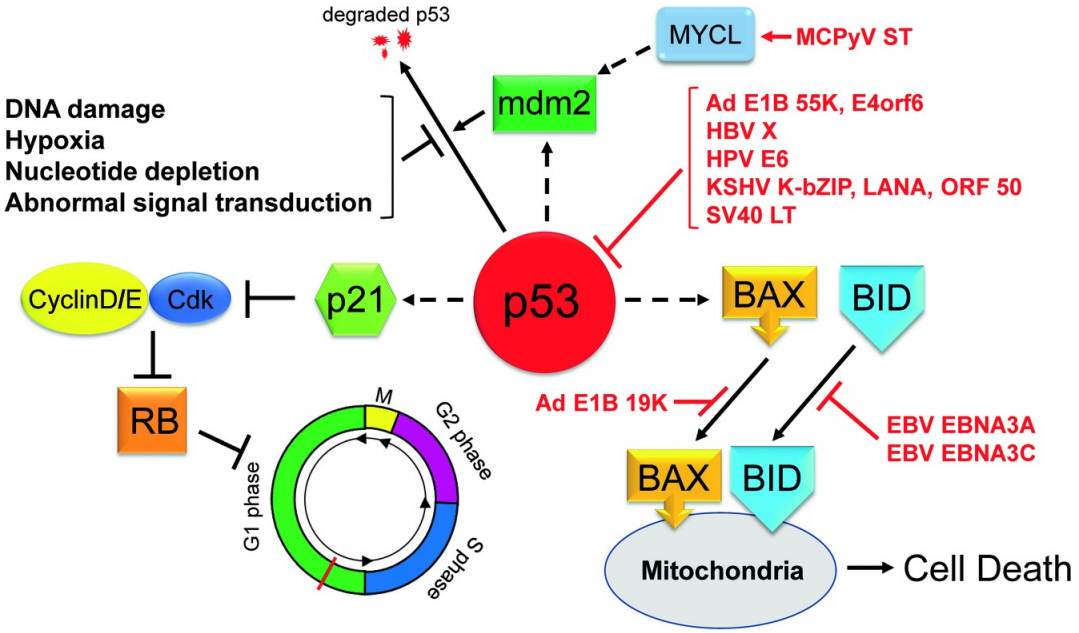

FIGURE 11.6 **Multiple DNA tumor viruses antagonize p53-mediated growth arrest and apoptosis**. Under normal conditions, p53 is maintained at a low level by a feedback loop in which p53 stimulates transcription of the mdm2 gene, and the resulting increased levels of mdm2 lead to p53 degradation. Stress signals activate kinases that phosphorylate p53, thereby disrupting its interaction with mdm2 and preventing degradation. The resulting increased p53 levels stimulate transcription of cdki's such as p21, which inhibit the ability of cyclin-dependent kinases to phosphorylate RB, resulting in cell cycle arrest. p53 also stimulates the transcription of multiple cell death genes such as Bax, which cooperates with Bid to penetrate the mitochondrial membrane and induce cell death. The circle at the bottom left represents the cell cycle. As described in the text, the DNA tumor virus oncoproteins shown in red interfere with p53 expression or action.

All viruses with DNA genomes must deal with the cellular DNA damage response (DDR). In response to DNA damage, the MRN DNA repair complex binds to DNA ends, recruits DNA repair proteins to sites of damage, and activates the ATM serine-threonine kinase. Activated ATM phosphorylates p53, leading to p53 activation, p21 induction, and ultimately loss of RB phosphorylation and repression of E2F-dependent transcription. Replicating viral DNA itself can trigger the DDR.[299] Furthermore, aberrant activation of mitogenic signaling pathways by viral oncoproteins can induce unscheduled cellular DNA synthesis leading to a DDR, apoptosis, or cellular senescence.[95] The p53 and RB pathways play critical roles in these processes, which could ultimately limit the production of viral progeny particles. Therefore, neutralization of these tumor suppressor pathways by viral oncoproteins also interferes with checkpoints that arrest the cell cycle in response to DNA damage, thereby removing these barriers to tumorigenesis. Furthermore, when these checkpoints fail, the resulting inaccurate DNA repair coupled with unscheduled DNA synthesis fosters the accumulation of mutations, some of which further contribute to the erosion of growth control.

In the next two sections, we describe the action of some important viral oncoproteins. In the sections on specific carcinogenic human viruses, we mention putative viral oncogenes whose mechanism of action is less well defined.

Mechanism of RNA Virus Oncogene Action

Numerous retroviruses can cause tumors in animals. Oncogenic retroviruses can be divided into two broad classes, based on the speed of tumor induction. *Acute transforming* retroviruses like RSV induce tumors rapidly (often within weeks in experimental animals) and usually transform cells in culture. As noted above, these viruses carry oncogenes responsible for rapid and efficient tumorigenesis and cell transformation. *Nonacute* oncogenic retroviruses induce tumors in animals more slowly (over months) and do not carry oncogenes or transform cells in culture. Rather, they induce tumors by affecting expression of cellular proto-oncogenes or tumor suppressor genes.

Overview of Retroviral Oncogenes

In general, acute transforming retroviruses contain viral oncogenes that are derived from cellular protooncogenes (Table 11.2). These oncogenes are inserted in various places in the retroviral genome and usually cause a viral replication defect by disrupting genes involved in viral replication (Fig. 11.1A). Cellular proteins encoded by protooncogenes, which generally play positive roles in stimulation of cell growth or division, are tightly regulated. The viral oncoproteins often differ from their parent cellular proteins by mutations and/or fusion to a retroviral replication protein, which release the tight regulatory control imposed on the normal cellular homologs, thereby activating the viral proteins compared to their cellular counterparts.

TABLE 11.2 Selected retroviral oncogenes and their protein products[a]

Oncogene	Retrovirus[b]	Oncoprotein[c]	HUGEN[d]	Identity
Growth factor				
sis	Simian sarcoma virus	$p28^{env-sis}$	*PDGFB*	Platelet-derived growth factor B chain
Receptor tyrosine kinase				
erbB	**AEV-ES4**	**$gp65^{erbB}$**	***EGFR***	**Epidermal growth factor receptor**
fms	**McDonough FeSV**	**$gp180^{gag-fms}$**	***CSF1R***	**Colony-stimulating factor 1 receptor**
sea/ron	**S13 AEV**	**$gp160^{env-sea}$**	***MST1R***	**Macrophage-stimulating protein receptor**
kit	**Hardy-Zuckerman-4 FeSV**	**$pp80^{gag-kit}$**	***KIT***	**Stem cell factor receptor**
ros	**UR2 ASV**	**$p68^{gag-ros}$**	***ROS1***	
eyk	Avian retrovirus RPL30	$gp37^{eyk}$	*MERTK*	Gas6 receptor
Cytokine or hormone receptor				
mpl	**Myeloproliferative leukemia virus**	**$p31^{env-mpl}$**	***MPL***	**Thrombopoietin receptor**
erbA	**AEV-ES4**	**$p75^{gag-erbA}$**	***THRB***	**Thyroid hormone receptor**
Lipid kinase				
p3k	**ASV16**	**$p150^{gag-p3K}$**	***PIK3CA***	**Catalytic subunit of PI3K**
Ubiquitin ligase				
cbl	**Cas NS-1 MuLV**	**$p100^{gag-cbl}$**	***CBL***	
G protein				
H-ras	**Harvey MSV**	**$p21^{H-ras}$**	***HRAS***	
K-ras	**Kirsten MSV**	**$p21^{K-ras}$**	***KRAS***	
Adaptor protein				
crk	**CT10, ASV-1**	**$p47^{gag-crk}$**	***CRK***	
Nonreceptor tyrosine kinase				
src	**Rous sarcoma virus**	**$pp60^{src}$**	***SRC***	
abl	**Abelson MuLV**	**$p160^{gag-abl}$**	***ABL1***	
fps/fes	**Fujinami ASV**	**$p130^{gag-fps}$**	***FES***	
fgr	Gardner-Rasheed FeSV	$p70^{gag-actin-fgr}$	*FGR*	
yes	Y73 ASV	$p80^{gag-yes}$	*YES1*	
Serine-threonine kinase				
akt	**AKT8 MuLV**	**$p105^{gag-akt}$**	***AKT1***	
mos	Moloney MSV	$p37^{env-mos}$	*MOS*	Cytostatic factor component
raf	**3611-MSV**	**$p75^{gag-raf}$**	***RAF1***	
Transcription factor				
jun	**ASV17**	**$p65^{gag-jun}$**	***JUN***	**AP-1 subunit**
fos	**Finkel-Biskis-Jenkins MSV**	**$p55^{fos}$**	***FOS***	**AP-1 subunit**
myc	**MC29 avian myelocytoma virus**	**$p100^{gag-myc}$**	***MYC***	
myb	**AMV BAI A**	**$p45^{myb}$**	***MYB***	
ets	AMV-E26	$p135^{gag-myb-ets}$	*ETS1*	
rel	**Avian reticuloendotheliosis virus T**	**$p64^{rel}$**	***REL***	**NFκB subunit**
maf	**Avian retrovirus AS42**	**$p100^{gag-maf}$**	***MAF***	
ski	SKV ASV	$p110^{gag-ski-pol}$	*SKI*	

[a]Oncogenes (or closely related oncogenes) mutated or overexpressed in some human cancers are shown in bold.

[b]In cases where different viruses encode closely related oncogenes, only a single virus is listed as an example.

[c]The nomenclature of viral oncoproteins refers to basic structural data: p, protein; gp, glycoprotein; pp, phosphoprotein; the numbers designate the molecular weight in kilodaltons; the superscript indicates the genes from which the coding information is derived in a 5′-3′ direction. These oncoproteins are frequently fused to a viral replication protein: *gag*, *pol*, or *env*.

[d]Name of the human gene according to the HUGO gene nomenclature committee.

Abbreviations: AEV, avian erythroblastosis virus; AMV, avian myeloblastosis virus; AP-1, activator protein-1; ASV, avian sarcoma virus; FeSV, feline sarcoma virus; NF-κB, nuclear factor-κB; MSV, murine sarcoma virus; MuLV, murine leukemia virus; PI3K, phosphotidylinositol 3-kinase.

The product of the *src* oncogene was the first oncoprotein characterized biochemically. *src* encodes a 60-kD phosphoprotein designated $pp60^{v\text{-}src}$ that phosphorylates tyrosine residues in proteins.[35,70,149] $pp60^{v\text{-}src}$ was the first tyrosine kinase discovered, and it phosphorylates many cellular protein substrates linked to oncogenesis. The kinase domain of $pp60^{v\text{-}src}$ is located downstream of two short protein domains known as "*src* *h*omology" (SH) domains (Fig. 11.1B): SH2 domains bind phosphotyrosine residues in specific sequence contexts in their target proteins, while SH3 domains bind proline-rich protein segments.[170,199] SH2 and SH3 domains are important protein-protein interaction motifs that are also present in many cellular proteins.

In viral oncoproteins, mutations may disrupt inhibitory elements present in the cellular protein. For example, pp60$^{c\text{-}src}$ has a C-terminal regulatory domain that contains a tyrosine residue at position 527 (Y^{527}). When Y^{527} is phosphorylated, it binds the SH2 domain in the same molecule of pp60$^{c\text{-}src}$, resulting in a closed conformation in which the kinase is inactive.[305] Dephosphorylation of Y^{527} displaces the SH2 domain and activates *src* kinase.[73] Of note, the viral protein pp60$^{v\text{-}src}$ contains a frameshift mutation that removes Y^{527} and as a consequence is constitutively in the open, kinase-active configuration (Fig. 11.1B).[278,281] Other viral oncoproteins contain amino acid substitutions that disrupt regulatory mechanisms (eg, the *v-ras* proteins) or are fused to viral proteins so that oncogenic activity is enhanced (eg, the *v-abl* protein). In some cases, the viral oncogene product is identical to the protooncogene product but is overexpressed from the retroviral genome (eg, the *v-myc* protein).

Retroviral Oncoproteins as Components of Signal Transduction Pathways

As described in an earlier section, untransformed cells in culture require growth factors that bind to cell surface receptors and trigger signaling cascades. Genes encoding many components of these signal transduction pathways have been captured by acutely transforming retroviruses (Fig. 11.3 and Table 11.2). Indeed, many of these components were first identified as retroviral oncogene products.

The *v-sis* oncogene encodes a version the PDGF B chain and can transform only cells that express the PDGF receptor, providing a molecular explanation for the cell-type specific action of some oncogenes.[60,210] Other retroviral oncogenes encode activated versions of RTKs. The *v-erbB* oncogene encodes a mutant EGF receptor that lacks the extracellular EGF binding domain and an autoinhibitory domain at the C-terminus.[88,141] As a result, the *v-erbB* protein signals in a constitutive, EGF-independent manner. Other constitutively activated RTKs encoded by acute transforming retroviruses include the stem cell factor receptor (*v-kit*) and the colony-stimulating factor 1 receptor (*v-fms*),[148,254,316] and the *v-cbl* oncogene encodes a ubiquitin ligase that regulates RTK abundance.[157] The *v-mpl* oncogene encodes a constitutively active version of the thrombopoietin receptor.[82,160] The ability of oncogenes to activate or serve as activated growth factor receptors provides a simple explanation for the reduced serum requirements of transformed cells.

Other oncogenes encode proteins that bind to RTKs or transmit signals from them. The *src* family are nonreceptor tyrosine kinases that mediate signaling from activated RTKs. In addition to *v-src* itself, the *src* family members *v-fps*, *v-fes*, and *v-yes* were captured by other acute transforming retroviruses.[129] An important lipid kinase is phosphoinositol 3′ kinase (PI3K), which delivers survival and proliferative signals to cells upon RTK activation. The gene encoding PI3K was captured as the *v-p3k* oncogene.[51] The *v-crk* oncogene encodes a receptor-associated adaptor protein that lacks enzymatic activity but contains SH2 and SH3 domains that assemble proteins into signal transduction complexes with RTKs.[197] The *v-abl* oncogene encodes a cytoplasmic tyrosine kinase that is activated by fusion to the retroviral gag protein.[302]

Other retroviral oncoproteins are cytoplasmic kinases that phosphorylate proteins on serines or threonines as part of the signal transduction cascade initiated by growth factor binding. These include proteins encoded by *v-raf*,[156,231] *v-mos*,[250] and *v-akt*[21] that ultimately lead to phosphorylation and nuclear translocation of mitogen-activated protein kinases.[171,313] v-*akt* encodes a homologue of Akt, a key kinase in downstream signaling from PI3K.[21,51] For the viral oncogenes that encode kinases, unregulated enzyme activity or overexpression causes overactive signaling through the corresponding signal transduction pathways.

v-Hras and *v-Kras* encode cytoplasmic guanosine triphosphatases (GTPases) that bind guanosine diphosphate [GDP] or guanosine triphosphate [GTP]. The GTP-bound form of Ras, which is generated by RTK activation, triggers signals that result in cell proliferation (eg, by binding and activating Raf kinase), but tras proteins are inactive when bound to GDP.[30,100,190] Active GTP-bound Ras cycles back to the inactive form through the action of its intrinsic GTPase activity, which is stimulated by GTPase activating proteins (GAPs).[285] The v-H*ras* and v-K*ras* proteins contain missense mutations at key residues that reduce the ability of GAP to stimulate GTP hydrolysis.[285] As a consequence, the v-*ras* proteins accumulate in the active, GTP-bound form and signal constitutively.

Many genes first identified as retroviral oncogenes are also mutated in sporadic human cancer (Table 11.2). In some cases, the specific genetic alterations in human cancer closely parallel the activating mutations in the retroviruses themselves. Drugs targeting the protein products of cellular protooncogenes can successfully treat human cancers containing mutations that activate Abl, EGF receptor, ErbB2 (an EGF receptor family member), PDGF α receptor, stem cell factor receptor, PI3K, and B-Raf.

Nuclear Retroviral Oncoproteins

Some retroviral oncogenes encode versions of cellular transcription factors. The *v-myc* oncogene is derived from *c-myc*, which encodes a protein (Myc) involved in early responses to mitogenic stimuli and binds directly to promoters/enhancers of target genes.[289] Early after a mitogenic stimulus, levels of Myc transiently rise, leading to activation of some genes, such as those encoding E2F and cyclin D, and repression of other genes including those encoding cdki's.[96] *v-myc* transforms cells because it is constitutively overexpressed from the retroviral long terminal repeat (LTR), which contains transcriptional regulatory elements. The *v-myb* oncogene is derived from *c-myb*, which encodes a transcription factor important in hematopoietic cell development.[24,128] Two additional interesting retroviral oncogenes are *v-fos* and *v-jun*, which encode mutant subunits of AP-1.[29,232] In normal cells, early after mitogenic stimulation, Fos-Jun heterodimers form and activate promoters/enhancers containing AP1-sites, leading to expression of target genes important for cell division and growth.[205] The *v-jun* and *v-fos* proteins contain structural alterations, such as a deletion that removes an auto-inhibitory segment from *c-jun*, resulting in oncogenic activation.[32]

The *v-erbA* protein is a dominant negative form of the thyroid hormone receptor-α, a sequence-specific DNA binding protein.[243,298] *v-ErbA* inhibits transcription of THR-α–responsive genes and blocks erythroid cell differentiation, resulting in enhanced proliferation of undifferentiated erythroid progenitors.[76] The *v-rel* oncogene encodes a mutant subunit of the nuclear factor-κB (NFκB) transcription factor family important for B-cell development.[117] Point mutations and fused sequences from retroviral *env* activate *v-rel*.[280]

Although most retroviral oncogenes were acquired from the host cell genome, some retroviral oncogenes are derived from viral envelope proteins. For example, the oncoprotein of Friend spleen focus forming virus, designated gp55, is a deleted form of a retroviral *env* protein.[65] gp55 binds the erythropoietin receptor (EpoR),[184] leading to constitutive downstream signaling and oncogenic transformation.[65]

Insertional Activation of Cellular Proto-oncogenes by Nonacute Transforming Retroviruses

Prototype nonacute oncogenic retroviruses include avian leukosis virus (ALV) and various murine leukemia viruses (MuLVs). These viruses lack viral oncogenes and do not transform cultured cells, and tumors arise in animals many months after infection. Most nonacute retroviruses induce tumors by transcriptional activation of cellular proto-oncogenes by the integrated proviral genome. Pioneering experiments investigating tumorigenesis by these viruses were conducted on chicken B-cell lymphomas induced by ALV. DNA from many of these tumors contained the ALV provirus inserted into the same chromosomal region in independent tumors, adjacent to the *c-myc* proto-oncogene (which was first identified as the oncogene in an acute transforming retrovirus).[142,253] This insertion placed *c-myc* under control of the strong promoter/enhancer in the proviral LTR, resulting in increased transcription of *c-myc* (Fig. 11.7). The viral LTR can act as either a promoter or an enhancer to cause transcriptional activation of protooncogenes. Murine leukemias induced by M-MuLV also commonly result from activation of *c-myc*.[251] Thus, *c-myc* can be activated either by transduction by acute transforming retroviruses or by proviral insertion by nonacute transforming retroviruses.

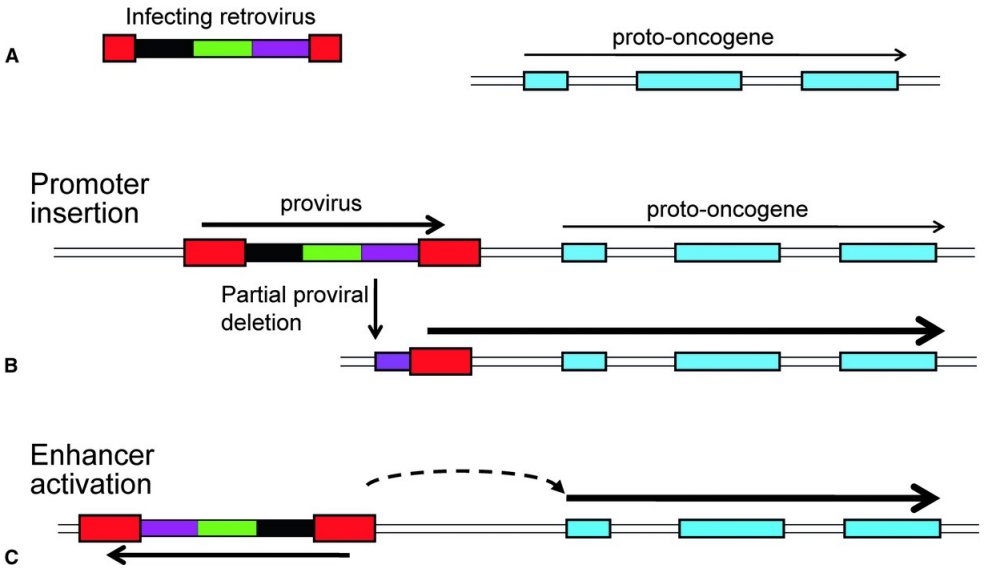

FIGURE 11.7 **Insertional activation of cellular proto-oncogenes**. Color scheme as in Figure 11.1A. **A**. Infecting retrovirus is shown at left and target protooncogene with three exons (*blue*) is shown at right. The *arrow* indicates the direction of transcription, and the thickness of the arrow represents the level of expression. **B**. Promoter insertion mechanism. After insertion of the provirus upstream of the proto-oncogene in the same transcriptional orientation, a portion of the provirus is often deleted, activating the right viral LTR to drive increased transcription of the proto-oncogene. **C**. Enhancer activation mechanism. After insertion of the provirus in the opposite orientation as the protooncogene (or downstream of the oncogene, not shown), the transcriptional enhancer in the viral LTR drives increased transcription of the proto-oncogene.

Proviral insertion can also activate other proto-oncogenes. For example, the mouse mammary tumor virus (MMTV) activates *int-1* (later renamed *wnt-1* after its *Drosophila* homolog[234]). *Wnt-1* protein is an important molecule in the *Wnt-β-catenin* signal transduction pathway, which is mutated in a wide variety of human cancers, most notably colorectal adenocarcinoma.[37] MuLV insertion can activate genes encoding other important cell cycle regulators including cyclin D2, DNA binding proteins of the *ets* family, and the macrophage colony stimulating factor. The LTRs of the M and F strains of MuLV activate different protooncogenes in T-cell lymphomas and erythroid leukemias, respectively, reflecting the different signaling pathways important for transformation of different cell types.

The genomes of many animals contain complete or partial integrated retroviral genomes, known as endogenous retroviruses. Certain strains of mice develop leukemia at a high frequency due to spontaneous activation of these endogenous viruses, which in turn infect new cells and insertionally activate cellular proto-oncogenes.[71] Recombination can also occur between exogenous infecting MuLVs and endogenous proviruses to generate recombinant viruses with altered tropism or heightened oncogenicity.[273]

In addition to LTR-mediated activation of proto-oncogene transcription, related mechanisms of oncogene activation also involve integration of proviral DNA into the cell genome. For example, proviral insertion can introduce new splice sites or transcriptional elements resulting in the synthesis of aberrant proteins or disrupt microRNA-mediated downregulation of cellular proto-oncogenes. Finally, proviruses can inactivate p53 or NF-1 by inserting into the coding region of these tumor suppressor genes.[22,178]

Mechanism of DNA Virus Oncogene Action

Unlike most retroviral oncogenes, DNA tumor virus oncogenes were not captured in the recent past from the host cell genome but rather are intrinsic viral genes that usually have been part of the viral genome for millions of years of viral evolution and play essential roles in virus replication or persistence (Table 11.3). In many cases, these genes encode proteins that mobilize the cellular DNA replication machinery to support the production of many progeny viral DNA genomes, so it is not surprising that increased cell proliferation can result from sustained expression of these proteins. Most DNA virus oncoproteins function by binding to cellular proteins and modulating their activities (Table 11.3). DNA tumor viruses often encode more than one oncoprotein that cooperate to transform cells. Some DNA virus oncoproteins stimulate cellular mitogenic signal transduction pathways, whereas others inactivate cellular tumor suppressor pathways (Figs. 11.4, 11.5, and 11.6). Still others impair immune surveillance against tumor virus replication and cancer (eg,[233]). It is striking that diverse DNA tumor viruses modulate a limited number of common cellular targets, implying that there are relatively few crucial nodes that regulate cell proliferation and survival (Table 11.4).

TABLE 11.3 Selected DNA virus oncoproteins and their major targets

Viral Oncoprotein	Cellular Target
Adenovirus E1A	RB family members, p300/CBP, CtBP
Adenovirus E1B 19K	Bak, Bax
Adenovirus E1B 55K	p53
Adenovirus E4orf6	p53
Bovine papillomavirus E5	PDGF β receptor
Epstein-Barr virus EBNA2	RBP-Jκ/CBF1, glycogen synthetase kinase
Epstein-Barr virus LMP1	Tumor necrosis factor signaling components, PI3K
Epstein-Barr virus LMP2A	*src* family members
Hepatitis B virus X protein	p53
Human papillomavirus E5	EGF receptor
High-risk human papillomavirus E6	p53, PDZ proteins, E6-AP, DNA repair and apoptosis machinery
High-risk human papillomavirus E7	RB family members, p21, p27, E2F1, cyclin-dependent kinases
KSHV K-bZIP	p53
KSHV LANA	p53, RB family members, chromatin proteins
KSHV ORF 50	p53
KSHV vCYC	Cyclin-dependent kinase 6
Polyomavirus large T antigen	p53 (not murine polyomavirus or MCPyV), RB family members
Polyomavirus middle T antigen	*src* family members, PI3K, PP2A, shc
Polyomavirus small T antigen	PP2A, MYCL (MCPyV)

EBNA, Epstein-Barr virus nuclear antigen; EGF, epidermal growth factor; KSHV, Kaposi sarcoma–associated herpesvirus; LMP, latent membrane protein; MCPyV, Merkel cell polyomavirus; PDGF, platelet-derived growth factor; PI3K, phosphotidylinositol 3-kinase; PP2A, protein phosphatase 2A; RB, retinoblastoma; vCYC, vCyclin.

TABLE 11.4 Common targets of DNA virus oncoproteins

p53	Large T antigen (some polyomaviruses, eg, SV40) High-risk HPV E6 Adenovirus E1B 55K and E4orf6 KSHV K-bZIP, LANA, and ORF 50 Hepatitis B virus X protein
RB family members	Large T antigen (all polyomaviruses) High-risk HPV E7 Adenovirus E1A KSHV LANA
Src family members	Polyomavirus middle T antigen EBV LMP2A
PI3K	Polyomavirus middle T antigen, EBV LMP1
PP2A	Polyomavirus small T antigen and middle T antigen

EBV, Epstein-Barr virus; HPV, human papillomavirus; KSHV, Kaposi sarcoma–associated herpesvirus; LANA, latency-associated nuclear antigen; LMP1, latent membrane protein 1; LMP2A, latent membrane protein 2A; PI3K, phosphotidylinositol 3-kinase; PP2A, protein phosphatase 2A; SV40, simian virus 40.

DNA Virus Oncoproteins that Activate Mitogenic Signaling Pathways

One of the first DNA virus oncoproteins characterized was the membrane-anchored middle T (MT) antigen of murine polyomavirus.[286] MT constitutively associates with a cellular tyrosine kinase that was later identified as none other than pp60$^{c\text{-}src}$, the product of the *c-src* protooncogene captured by RSV![74,93] MT binding activates the kinase activity of pp60$^{c\text{-}src}$, which then phosphorylates tyrosines in the large cytoplasmic domain of MT, thereby generating binding sites for cellular signaling proteins that contain SH2 domains such as PI3K and protein phosphatase 2A (PP2A) (reviewed in Ref.[245]) (Fig. 11.4). Because many of the proteins that bind MT also bind to the cytoplasmic domain of ligand-activated RTKs, MT mimics an activated RTK that delivers a constitutive mitogenic signal.

The bizarre bovine papillomavirus (BPV) E5 protein uses a related mechanism to transform cells. The shortest known oncoprotein, the E5 protein is only 44 residues long and extremely hydrophobic, essentially a free-standing transmembrane domain.[248] The E5 protein binds specifically to the transmembrane domain of an RTK, the PDG β receptor, causing receptor dimerization and sustained ligand-independent activation, which results in cell transformation (Fig. 11.4).[83,121,222,223] Thus, like the retroviral oncogene *v-sis*, the which encodes PDGF itself, the E5 protein causes constitutive activation of the PDGF receptor, but E5 uses entirely different biochemical interactions to drive receptor activation.

The E5 proteins from certain HPV types display weak transforming activity in cultured cells and contribute to cancer development in transgenic mice. The HPV16 E5 protein increases the sensitivity of the EGF receptor to EGF, and the EGF receptor is required for the growth-promoting effects of the HPV16 E5 protein in transgenic mice.[114,274] HPV16 E5 has also been reported to modulate the activity of vacuolar H^+-ATPase, act as a viroporin, and downregulate major histocompatibility antigens.

DNA Virus Oncoproteins that Inactivate Tumor Suppressor Pathways

All members of the *Polyomaviridae* encode a nuclear protein, large T antigen (LT), a multifunctional protein that consists of four folded domains separated by flexible unstructured regions: a J domain near the amino-terminus, a DNA-binding domain (OBD) that binds the origin of viral DNA replication, a zinc-binding domain, and an ATP-dependent helicase domain that, in some polyomaviruses, also binds p53.[224] The region between the J domain and OBD contains docking sites for several cellular proteins including an LXCXE motif that binds members of the RB family.

SV40 LT is a potent oncoprotein that transforms cells in culture and induces neoplasia in transgenic mice, primarily by inhibiting the activity of p53 and RB (Figs. 11.5 and 11.6).[1] LT activities that function directly in viral DNA replication are not required for transformation. In contrast, transformation is impaired by mutations that disrupt LT binding to RB proteins or p53. LT binds to RB-E2F repressive complexes and dislodges E2F from RB, resulting in de-repression of E2F-regulated genes and cell cycle entry. Energy for E2F displacement is derived from hsc70-mediated ATP hydrolysis stimulated by the LT J domain. Nearly all LT proteins contain a J domain and LXCXE motif, so this mechanism appears conserved.

LT action on RB is sufficient to stimulate cell proliferation, but this is accompanied by p53-dependent apoptosis. LT also binds and inactivates p53 so it is unable to bind the promoters of growth arrest genes like p21 or apoptosis inducers like PUMA, BID2, and BAX. The combined effects of stimulating proliferation by binding RB and blocking cell cycle arrest and apoptosis by binding p53 are major contributors to the transforming activity of LT. Unlike SV40 LT, Merkel cell polyomavirus (MCPyV) LT does not bind p53.[59,183] Rather, the p53 pathway is blocked by a small T antigen (ST), which consists of the same amino-terminal J domain as LT and a carboxy-terminal series of metal binding motifs. MCPyV ST associates with MYCL (the product of *L-MYC*, a member of the c-*myc* gene family) and prevents repression of MYC-regulated genes including the gene encoding mdm2 (Fig. 11.6).[58,214] The resulting increased levels of mdm2 stimulate the degradation of p53. SV40 ST is required for the transformation of some cell types. ST binds to the catalytic subunit of PP2A and alters its substrate specificity.[241]

Adenoviridae and *Papillomaviridae* oncoproteins also antagonize RB to stimulate proliferation and p53 to block apoptosis (Figs. 11.5 and 11.6). The major adenovirus oncogenes are E1A and E1B, and the major HPV oncogenes are E6 and E7. The protein products of these genes are

not enzymes; rather they act by binding to and modulating the activity of cell proteins. Human adenovirus type 5 encodes two E1A isoforms, 289R and 243R, both of which bind RB proteins via an LXCXE motif and stimulate E2F-dependent transcription and cell proliferation. RB binding displaces E2F so it can stimulate transcription. The high-risk HPVs E7 proteins also bind RB through an LXCXE motif and stimulate their degradation by the proteasome, thereby inhibiting their ability to block E2F-dependent transcription.

The proliferation induced by E1A or E7 is accompanied by p53-dependent apoptosis. Therefore, like polyomaviruses, HPV and adenovirus proteins antagonize p53. The adenovirus 5 E1B 55K protein binds p53 and disrupts its association with transcription factors. E1B 55K also acts in concert with the viral E4 ORF6 protein to stimulate p53 degradation. In addition, the E1B 19K protein mimics the cellular antiapoptotic Bcl-2 protein and sequesters BAX, thereby blocking apoptosis downstream of p53 signaling. The HPV E6 protein also binds to p53 and targets it for ubiquitination by the E3 ubiquitin ligase E6AP, resulting in p53 proteasomal degradation. Thus, cells expressing E1B or E6 are functionally devoid of p53.

Viral oncoproteins from three different DNA virus families, with different genome structures and infectious cycles, have converged on RB-E2F and p53 to manipulate cell proliferation and death (Table 11.4). However, these are not the only targets of these viral oncoproteins relevant to tumorigenesis. LT, E1A, E1B 55K, E7, and E6 all associate with multiple cellular proteins that control the DDR, innate immunity, or gene expression (Table 11.3). High-risk HPV E6, for example, has a short, carboxy-terminal motif that binds cell proteins containing PDZ domains, and HPV E7 binds cdk's and E2F1.[17] Mutational studies have linked some of these interactions to transformation in cell culture.

Herpesvirus Oncoproteins

Epstein-Barr virus (EBV) and Kaposi sarcoma–associated herpesvirus (KSHV) encode numerous oncoproteins. EBV expresses several nuclear antigens (EBNAs). EBNA2 is essential for immortalization of B lymphocytes[69,136] and in some settings cooperates with EBNA-LP to increase transcription of genes involved in cell proliferation or signaling.[193,266,294] EBNA2 is recruited to promoters by binding the cellular transcription factor RBP-Jκ, which also mediates signaling through the receptor for Notch, thus mimicking an active Notch receptor.[130,187] Consistent with this view, activated Notch can partially restore B-cell transforming activity to EBV EBNA2 mutants.[125,216] Expression of EBNA3A and EBNA3C is also necessary for B-cell immortalization. These proteins cooperate to silence expression of the cdk inhibitor $p16^{INK4A}$ and the proapoptotic protein Bim.[3,261,306] EBNA3C also cooperates with activated Ras to transform rodent cells in culture and inhibits apoptosis by binding to p53.[306]

Cell transformation and tumor formation by EBV are indirectly supported by EBNA1, which initiates viral DNA replication and ensures maintenance of the viral genome during cell division.[308] EBNA1 is also required to activate latency genes and been reported to alter signaling pathways including p53, TGF-b, and NFkB and to block cell death by upregulating the pro-survival gene, survivin.[191,288,303]

EBV LMP1 and LMP2A are both multipass membrane oncoproteins. LMP1 is essential for EBV-mediated transformation of human B cells and can transform cultured epithelial cells and fibroblasts.[154,161,162,201,220,293] In transgenic mice, LMP1 can induce epithelial and B-lymphocytic hyperplasia, which can progress to lymphoma.[175,194] LMP1 activates c-jun N-terminal kinase (JNK) and p38/MAPK pathways leading to activation of NF-κB, which is necessary for transformed cell growth.[40,97,98] In addition, LMP1 binds to several tumor necrosis factor (TNF) receptor–associated factors (TRAFs), receptor interacting protein (RIP), and a TNF receptor–associated death domain (TRADD) protein.[152-154,201] These proteins also bind to the pro-survival CD40 form of the TNF receptor. Thus, LMP1 mimics a constitutively active CD40 TNF receptor to deliver a pro-survival signal.[287] LMP1 also binds PI3K, which may contribute to its pro-survival effects by activating akt.[80] LMP2A is required to maintain viral latency and transforms epithelial cells in culture. It activates Src and Lyn leading to activation of the PI3K pathway.[111,239,249]

KSHV expresses latency-associated nuclear antigen (LANA), a large multifunctional protein required for the establishment and maintenance of the extrachromosomal viral genome.[10,72,147] LANA can transform cultured fibroblasts in cooperation with activated Ras and causes lymphocyte hyperplasia and lymphomas in transgenic mice.[104] LANA binds and inhibits p53 and RB and interacts with a number of transcription factors and chromatin-associated proteins such as histone H2A/B, Brd4, STAT3, and ATF4/CREB2.[14,186,212,297] In addition, LANA stabilizes β-catenin, which leads to increased expression of cyclin D and Myc.[110] LANA also upregulates telomerase expression and activates important signal transduction pathways including Wnt, Notch, PI3K, MAPK, and JAK/STAT, leading to the release of angiogenic factors.[176,202,291]

KSHV also encodes oncoproteins that are homologous to cellular proteins, including v-Cyclin or vCYC, which binds and activates CDK6 leading to RB phosphorylation and cell cycle entry. Unlike cellular cyclin D, vCYC is resistant to inhibition by cdki's.[54,279] KSHV also expresses vIRF3, a homolog of the cellular transcription factor IRF3 that promotes cell growth, interacts with p53, and downregulates p53-mediated transcription.[15,55,113] In addition, vIRF3 displaces the Myc inhibitory protein MM-1α, leading to Myc-mediated transcriptional activation.[192] The KSHV-encoded FLICE-inhibitory protein (vFLIP) stimulates B-cell proliferation and survival and induces lymphomas in transgenic mice.[11,64] vFLIP binds to and antagonizes IKKγ, resulting in NFkB activation.[56,119,276] In addition, KSHV encodes active homologues of several cellular chemokines and cytokines, such as v-IL6. Finally, KSHV expresses several microRNAs that have been implicated in cell transformation.

KSHV expresses a lytic program in some cancers, and several lytic viral genes have been reported to have oncogenic properties. These include ORF50 and K-BZIP, which interfere with p53 activity[133,215]; K1, which transforms cells and encodes a transmembrane glycoprotein that mimics signaling by the B-cell antigen receptor and activates the PI3K pathway[180,228,229]; and vGPCR, which encodes a constitutively active protein homologous to G protein–coupled receptors.[50] vGPCR activates various mitogenic and pro-survival signaling pathways, displays transforming activity in cultured cells, and can induce tumors in mice that resemble Kaposi sarcoma (KS).[8,45,198] Finally, KSHV encodes vBcl-2 and vIAP, lytic proteins that are homologous to Bcl-2 and survivin, respectively, that inhibit apoptosis and contribute to lymphoma formation in transgenic mice.[56,57,64,295]

Human Tumor Virology

Eight viruses are currently recognized as human carcinogens: human immunodeficiency virus (HIV) and seven tumor viruses: human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV), Epstein-Barr Virus (EBV), Kaposi sarcoma–associated herpesvirus

(KSHV), human T-cell leukemia virus type 1 (HTLV-1), and Merkel cell polyomavirus (MCPyV). These viruses cause almost 1.5 million cancers in humans each year, more than 10% of the worldwide cancer burden (Table 11.5).[81] The fraction of cases of specific cancers attributed to viruses ranges from about 10% (for gastric cancer and EBV) to essentially 100% (for cervical cancer and HPV) (Fig. 11.8). Virus-induced cancers preferentially occur in immunosuppressed individuals.

TABLE 11.5 Cancers associated with viral carcinogens[a]

Virus	Major Associated Cancers	Annual Cases[b]
Human papillomavirus (HPV)	Cervical carcinoma, other anogenital cancers, oropharyngeal and other head-and-neck carcinomas[c]	694 000
Hepatitis B virus (HBV)	Hepatocellular carcinoma	360 000
Hepatitis C virus (HCV)	Hepatocellular carcinoma, non-Hodgkin lymphoma	156 000
Epstein Barr virus (EBV)	Burkitt lymphoma, nasopharyngeal carcinoma, non-Hodgkin lymphoma, gastric carcinoma	157 000[d]
Kaposi sarcoma–associated herpesvirus (KSHV)	Kaposi sarcoma, primary effusion lymphoma, multicentric Castleman disease	42 000
Human T-cell leukemia virus type-1 (HTLV-1)	Adult T-cell leukemia/ lymphoma	4000
Merkel cell polyomavirus (MCPyV)	Merkel cell carcinoma	—[e]
Human immunodeficiency virus (HIV)	Various cancers	—[f]

[a]Viruses classified as carcinogens by the International Agency for Cancer Research of the World Health Organization or the National Toxicology Program of the United States Department of Health and Human Services.

[b]Infection-associated cases worldwide in 2018 as estimated by de Martel et al. (2020).[81]

[c]Only high-risk HPV (eg, HPV16 and HPV18) are associated with the cancers listed here. Other HPV types are associated with skin cancer, which is not included in this tabulation.

[d]This total does not include EBV-associated gastric cancers, which number in excess of 50 000 cases annually.

[e]MCPyV is thought to be responsible for several thousand deaths annually worldwide.

[f]HIV is classified as a human carcinogen but is not generally regarded as a tumor virus. Many cancers associated with HIV infection are caused by the tumor viruses listed in this table.

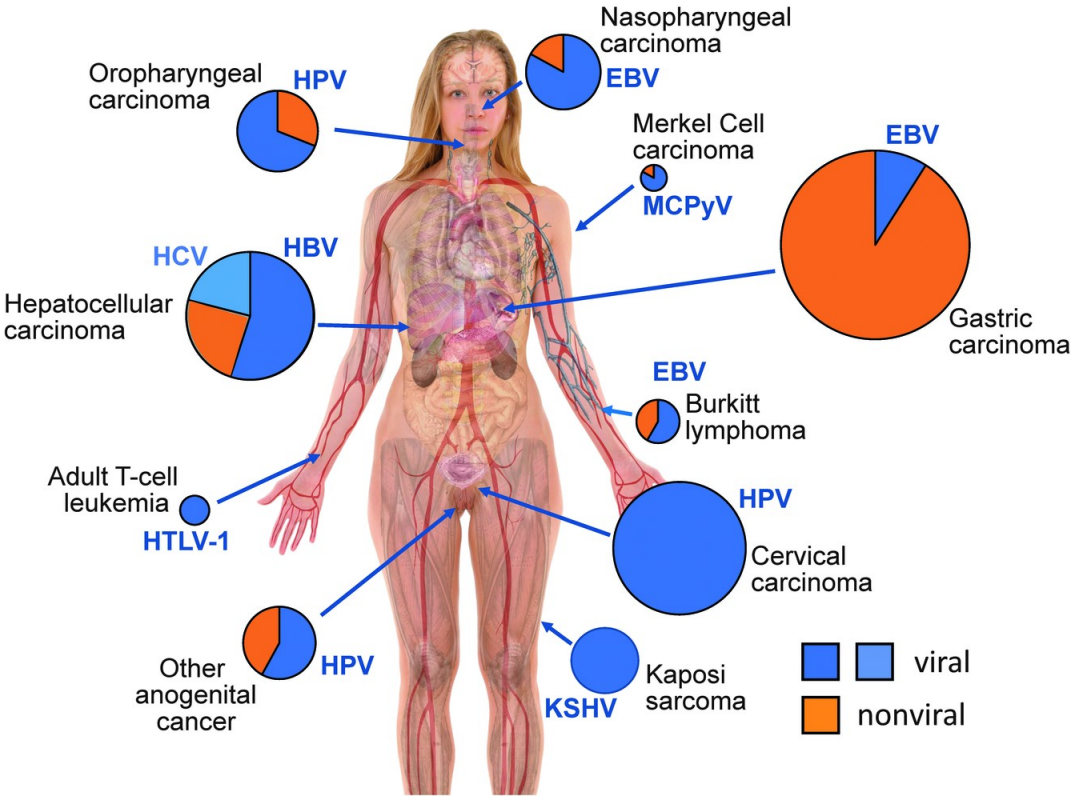

FIGURE 11.8 **Fraction of cancers caused by tumor viruses**. The sizes of the circles represent the incidence of the indicated cancers (data for males and females are aggregated), and the *blue pie* slices show the fraction that is attributable to the indicated virus. KSHV is also associated with 100% of primary effusion lymphoma and 50% of multicentric Castleman disease, two rare lymphoid malignancies.

Discovery of Human Tumor Viruses and Establishing Causality

The search for human tumor viruses was stimulated by the discovery of viruses that cause tumors in mammals.[301] This search took on special urgency in the 1960s with the startling discovery that some stocks of the poliovirus vaccine administered to tens of millions of people were contaminated with SV40, which induces tumors in hamsters.[94,118] Fortunately SV40 does not appear pathogenic in humans.

It took decades to identify and validate human tumor viruses, in part because there is no universal agreement on criteria needed to establish causality. This search was further complicated by several factors. Tumor viruses typically cause cancer in only a small fraction of infected people. In fact, some tumor viruses including EBV and MCPyV are ubiquitous, while their associated cancers are relatively rare. Virally induced cancers usually arise decades after the initial infection, which can obscure epidemiological links. Virus action is not sufficient for cancer. Rather, tumor viruses cooperate with cellular mutations, copy number variants, and epigenetic changes to induce tumors. Finally, for some viruses (eg, EBV and MCPyV), the cancer does not produce virus, while for others (eg, HBV and HCV), productive infection appears to be necessary for tumorigenesis. For HPV, benign tumors (warts) are a normal feature of productive infection, but the cancer cells do not produce virus. The presence of a virus (or a viral genome) in tumor tissue may merely signify that the tumor provides favorable cellular conditions for productive virus infection or latency. Thus, presence of viral DNA in cancer cells does not establish viral cause.

EBV, HPV, and MCPyV are very different viruses that cause distinct cancers, but they share common features that indicate a driver role in tumorigenesis. First, all or part of viral genome is present in all tumor cells and persists as cells proliferate. Second, the tumor cells express one or more viral oncoproteins that transform cells in culture, and continuous oncoprotein expression is required to maintain the transformed phenotype. Notably, tumors associated with EBV, HPV, and MCPyV have a mutational landscape and gene expression profile consistent with the known actions of the viral oncoproteins, and in MCPyV-associated skin cancer, EBV-associated gastric cancer, and HPV-associated head-and-neck cancer, the mutation signature of virus-associated tumors differs from the signature of the same type of tumor lacking viral genomes.

Tumors induced by KSHV, HCV, and HBV often contain cells undergoing productive virus infection. KSHV expresses genes during latency or productive infection that transform cells in culture and/or induce tumors in transgenic mice, consistent with a driver role for KSHV in these tumors. HCV does not encode proteins that are clearly oncogenic and viral genes are not expressed in all tumor cells, suggesting that productive infection plays an indirect role in hepatocellular carcinoma (HCC) by inducing liver damage leading to an accumulation of cellular mutations that drive tumorigenesis. The relative contribution of HBV-induced liver damage and viral gene action to cancer development is not clear.

If a virus is causative, treatments that reduce or eliminate infection should decrease cancer risk. This has been observed with HBV and HPV vaccines and with HCV antiviral therapy, but even this finding does not necessarily indicate a direct carcinogenic role for the virus. For example, HIV antiviral therapy has greatly reduced the incidence of KS, but the proximate causative agent is KSHV, not HIV. Thus, although HIV is classified as a carcinogen, it is not considered a tumor virus. Given the diversity of viruses and carcinogenic mechanisms, we propose that it is not appropriate to demand a rigid set of criteria akin to Koch's postulates to infer causality. Rather, the accumulation of multiple, independent, and diverse pieces of evidence are required to establish causality (Table 11.6), and any one type of evidence may take on greater or lesser importance depending on the particular virus and the particular cancer.

TABLE 11.6 Lines of evidence implicating a virus in cancer causation[a]

	HPV	MCPyV	EBV	KSHV	HBV	HCV	HTLV-1	HIV
Prior infection with a specific virus is epidemiologically linked to increased risk of a specific cancer	+	+	+	+	+	+	+	+
At least part of the viral genome is present and expressed in all or a subset of cases of a specific type of cancer	+	+	+	+	+		+	
The virus or viral genes alone or in combination transform cells in culture	+	+	+	+	+	+	+	
Continuous viral gene expression in transformed cells is required for the transformed phenotype	+	+	+					
One or more viral genes with transforming activity is expressed in human cancer cells	+	+	+	+	+		+	
The virus or viral genes induce cancers in laboratory animals, preferably the same type of cancer the virus is associated with in humans	+	+	+	+	+	+	+	
Mutational landscape and gene expression profile in cancer is consistent with known functions of the viral oncoprotein(s)	+	+						
Mutation signature of virus-associated cancers differs from that of non-virus-associated cancers of the same type	+[b]	+	+[c]					
Antiviral therapy or prophylactic vaccination reduce risk of specific virus-associated cancer	+			+	+	+		+

[a]+ indicates that the listed virus meets the criterion. Many of these criteria will not be met by a hit-and-run viral carcinogen.

[b]For oropharyngeal cancer.

[c]For gastric cancer.

Each of the known human tumor viruses was associated with cancer by different approaches,[301] but the detection of viral nucleic acids in tumor cells has become the standard route of discovery of new tumor viruses, resulting in the discovery of KSHV and MCPyV by Chang and Moore and their colleagues. However, it is also possible that a virus could trigger carcinogenesis and then depart without leaving residual viral genes or gene products in the cancer cells. This hypothetical mechanism is termed “hit-and-run” carcinogenesis.[209] For example, animal studies suggest that skin carcinogenesis triggered by cutaneous HPV types may involve a hit-and-run mechanism in which the virus potentiates ultraviolet light–mediated mutagenesis but is not needed to maintain the cancerous state.[292] DNA sequencing to identify viral genomes in cancer cells will not identify instances of hit-and-run carcinogenesis.

Principles of Viral Carcinogens

Several unique features of tumor viruses and HIV have important implications for human cancer (Fig. 11.9).[85] The direct action of classic carcinogens such as chemical mutagens or radiation is restricted to the individual exposed to the carcinogen, but infectious carcinogens can be transmitted from one host to another, often to many other hosts and at a great distance from the initial exposure. Unlike chemical and physical carcinogens, viral carcinogens can amplify, mutate, evolve, and spread. Mutations might affect virus transmissibility or carcinogenicity or the susceptibility of the infection or the resulting cancer to prevention or treatment. Viruses can also specifically modulate the immune system, which can in turn can affect virus replication, spread, or pathogenicity. Notably, exposure to viral antigens can generate an immune response that inhibits subsequent infection. Viruses can also influence oncogenesis and cancer therapy in many ways in addition to by acting as direct carcinogens. For example, infection may induce or suppress a DNA damage or immune response or alter the metabolism of xenobiotics, which could affect cancer development or response to treatment. Most importantly, prevention or treatment of the underlying infection can reduce cancer risk. Indeed, the most important laboratory-based advances in cancer prevention in recent decades are interventions against virus-associated cancers: vaccines that prevent HBV or HPV infection and drugs that cure HCV infection and suppress HIV, HBV, and herpesvirus replication. In addition, HBV, HCV, HTLV-1, and HIV have been removed from the blood supply in much of the world, and public health measures such as needle exchange programs and encouragement of safe sex practices have reduced transmission of some tumor viruses and HIV. Widespread use of these and future preventive and therapeutic approaches will significantly reduce the worldwide cancer burden.

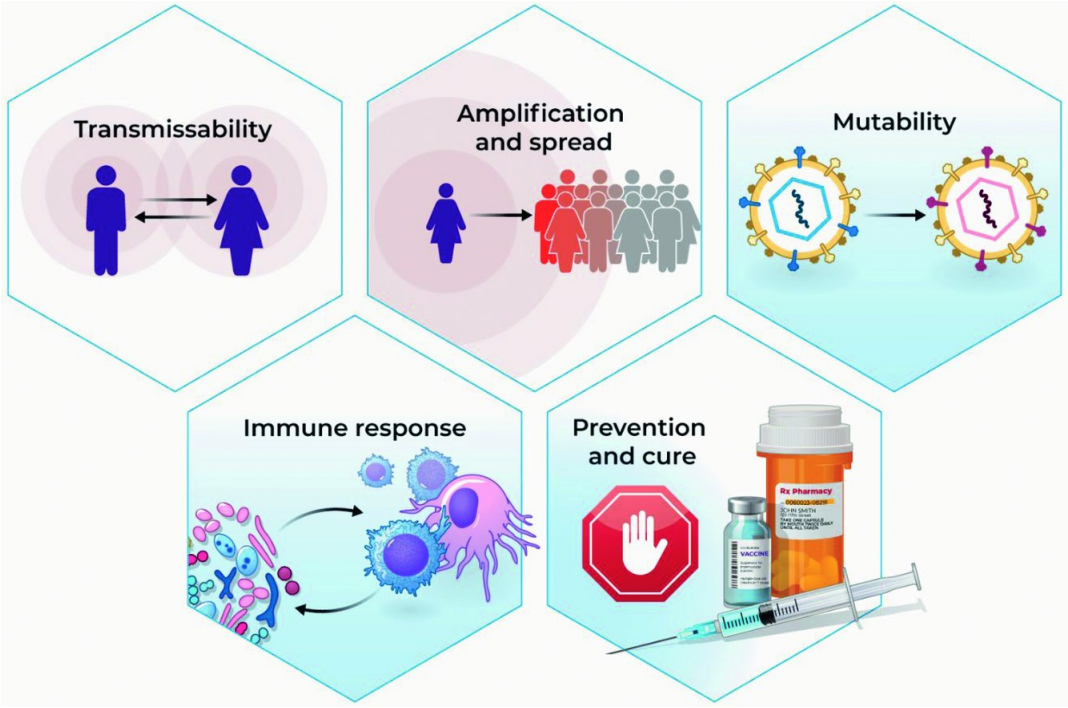

FIGURE 11.9 **Characteristic features of viral carcinogens**. The figure shows schematic diagrams of the key ways viral carcinogens differ from classic chemical and physical carcinogens. See text for details. (From DiMaio D, Emu B, Goodman AL, et al. Cancer microbiology. *J Natl Cancer Inst*. 2022;114(5):651-663. Reproduced by permission of Oxford University Press.)

Human Tumor Virus Action

Carcinogenic viruses employ diverse mechanisms to induce cancer, ranging from HPV and MCPyV, whose carcinogenic activity is dependent on viral oncogenes, to HIV with no oncogenes at all (Fig. 11.10).[112] HPV, MCPyV, EBV, and KSHV express strong, well-characterized oncoproteins that stimulate cell proliferation, inhibit cell death, and/or cause genetic instability, and they deploy immune evasion strategies that may promote tumor development. In contrast, HBV, HCV, and HTLV-1, lack clearly defined oncogenes (although each encodes proteins that can induce transformation or tumorigenesis when overexpressed). It is thought that the main contribution of HIV to carcinogenesis is through immunosuppression.[131]

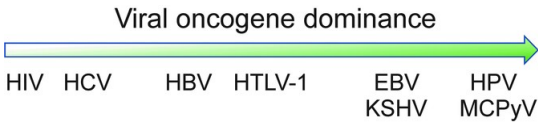

FIGURE 11.10 **Viral oncogenes and human cancer**. Schematic diagram illustrating the different degrees to which carcinogenic viruses depend on viral oncogenes. Some viruses such as HPV and MCPyV encode strong oncogenes that play the dominant role carcinogenesis, whereas HIV contains no oncogenes and appears to induce tumors indirectly by inhibiting immune function. Other carcinogenic human viruses display intermediate degrees of oncogene dependence.

For all human tumor viruses, only a few percent of infected people develop cancer. Some germline sequence variants in humans are associated with higher risk of developing cancer in response to certain virus infections, but even in people carrying these variants, cancer risk is low. However, individuals with primary immunodeficiency display a higher incidence of many cancers, including virus-induced cancers. Furthermore, the development of human viral cancers typically follows persistent or latent infection (often of decades-long duration) and preferentially occurs in the setting of immune suppression or chronic inflammation. These features of viral carcinogenesis suggest that additional, rare stochastic events such as the accumulation of specific mutations are required for cancer formation in humans. Consistent with the view that mutations are key, cancers induced by viruses contain many mutations, although usually not as many mutations as in nonviral tumors.[42,311] The specific cellular genes mutated in virus-induced tumors and the specific types of mutations are often distinct from mutations in sporadic cancers. Notably, the host responses to infection, including chronic inflammation or DNA damage, can induce mutations that can cooperate with the direct carcinogenic effects of the viral oncoproteins.

An important aspect of carcinogenesis initially discovered by studies with tumor viruses is “oncogene addiction,” namely the concept that ongoing expression of the transformed or tumorigenic state requires continuous oncogene expression. This was first shown with temperature-sensitive mutants of RSV, where the transformed phenotype was extinguished at the nonpermissive temperature.[195] Oncogene addiction suggests that inhibition of viral oncogene action may provide novel cancer treatment opportunities.

Finally, 85% of virus-induced cancers occur in low- and middle-income countries (LMICs), where they cause significant mortality. Many factors contribute to this disparity including the high prevalence of HIV and HBV in sub-Saharan Africa and Asia, respectively. Cancer outcomes in LMICs are driven in large part by environmental and behavioral risk factors, inadequate preventative and early detection programs (such as Pap smear screening for cervical cancer), and limited access to vaccines and treatment (*eg*, see Ref.[2]). In addition, poor nutrition is likely to impair host defense against infections and cancer development.

Human Papillomaviruses

In terms of the number of cancer cases, the most important human tumor viruses are the HPVs, a large group of nonenveloped viruses that contain an ~8000 base-pair double-stranded DNA genome encoding ~10 proteins.[317] HPVs are transmitted via direct contact with infected skin or mucosal tissue and infect billions of people worldwide. Infection causes papillomas or warts, benign epithelial cell tumors of the skin and mucous membranes. Although warts are usually self-limited, genital warts (condyloma acuminata) and laryngeal papillomas can be serious medical conditions.

In aggregate, 5% of human cancer is thought to be triggered by HPV infection.[246] High-risk mucosal strains of HPV such as HPV16 and HPV18, which were first discovered in cervical cancer DNA samples by zur Hausen and colleagues,[33,91] are responsible for virtually all cancers of the uterine cervix (the most common HPV-associated cancer) and a substantial fraction of vulvar, vaginal, anal, penile, and oropharyngeal cancer (Fig. 11.8). Cervical cancer is caused by numerous types of high-risk HPV, but a single high-risk type, HPV16, is responsible for the great majority of other HPV-associated anogenital and oropharyngeal cancers.[116] In addition to HPV, smoking, exogenous estrogen, and other infections also contribute to cervical cancer risk. In high-income areas of the world, robust Pap smear cervical cancer screening and treatment programs have greatly reduced the prevalence of cervical cancer, but it is still a major cause of death in LMIC.[2] Genetically distinct HPV types are also implicated in nonmelanoma skin cancer, particularly in immunosuppressed individuals.[139]

Most people infected with HPV do not develop HPV-associated cancer, and the cancers that do arise occur in the setting of persistent HPV infection years or decades after initial infection, presumably because HPV-infected cells must accumulate additional mutations to form cancers.[90,150] Indeed, cervical cancer has a high tumor mutational burden with tumors frequently containing many chromosomal translocations and somatic mutations.

Most cervical cancers continuously express the high-risk HPV E6 and E7 oncogenes, usually from viral DNA integrated into the host cell genome. Viral DNA integration may enhance expression of the viral oncogenes and affect expression of nearby cellular genes including *c-myc*.[221,296] As described in an earlier section, the E6 and E7 oncoproteins, among other activities, inactivate the p53 and RB tumor suppressor pathways, respectively, resulting in growth stimulation and genomic instability, which presumably contributes to the mutational burden of these cancers. The oncoproteins from the high-risk HPV types are more active at inactivating tumor suppressor pathways than are the proteins from the low-risk types, such as HPV6 and HPV11, which cause genital warts, not cancer. The HPV E5 oncogene is expressed in some but not all cervical cancers, and its role in human carcinogenesis is unclear. In addition, HPV deploys a number of immune evasion strategies that allow persistent HPV infection, which is an important risk factor for cancer development.[126] For example, high-risk HPV E7 binds directly to STING, a mediator of foreign DNA sensing, and inhibits interferon induction.[179]

HPV-associated squamous cell carcinoma of the oropharynx and tonsils occurs predominantly in males and is less aggressive than classic head-and-neck cancer, which is caused by tobacco and alcohol use.[4] It is not clear if the same pathogenic mechanisms are responsible for HPV-associated oropharyngeal and cervical cancer. Because only a fraction of oropharyngeal cancers contains HPV DNA, it is instructive to compare the mutations in HPV-negative vs HPV-positive head-and-neck cancer. These studies showed that p53 is mutated in over 80% of HPV-negative tumors, while nearly all HPV-positive tumors express wild-type p53. This is consistent with removal of p53 by the HPV E6 oncoprotein, thereby relieving the selective pressure for mutational inactivation of p53.

Repression of E6/E7 expression in cervical cancer cell lines or in HPV-induced cervical tumors in genetically engineered mice reactivates dormant tumor suppressor pathways and causes cellular senescence, apoptosis, or tumor regression.[123,124,155] Thus, continuous expression of the HPV oncogenes is required to maintain ongoing proliferation of the cancer cells, suggesting that strategies that interfere with E6/E7 expression or action may have an antitumor effect in patients with cervical cancer.[213]

Although no specific antiviral drugs target HPV, there are several highly effective prophylactic vaccines. The major HPV capsid protein, L1, can self-assemble to form noninfectious viruslike particles (VLPs) that elicit neutralizing antibodies.[63,168,315] L1 VLP vaccines protect against infection by the HPV types in the vaccine and reduce the risk of cervical cancer, generating high confidence that cervical carcinoma incidence will steeply decline in vaccinated populations in coming years.[172,181,263] All deployed HPV vaccines contain HPV16 (as well as other HPV types), so they should eventually prevent the great majority of HPV-associated anogenital and oropharyngeal cancer.[116] Unfortunately, however, vaccine coverage is suboptimal in much of the world, particularly in LMIC. Small clinical studies suggest that therapeutic vaccination with the E6 and E7 proteins can generate cytotoxic T cells that specifically kill cancer cells expressing these proteins.[167] The combination of vigilant screening and treatment of precursor lesions, prophylactic and therapeutic vaccination, and yet-to-be developed drugs that inhibit HPV replication or the progression or maintenance of HPV-associated cancers may lead to the eradication of cervical cancer.

Merkel Cell Polyomavirus

Merkel cell carcinoma (MCC) is a rare but aggressive neuroendocrine skin cancer that is frequently associated with immunosuppression and sunlight exposure.[19,101] MCPyV was discovered because its DNA is clonally integrated into the host cell of about 80% of MCC specimens.[107] Clonal viral DNA integration implies that virus infection occurred early during tumorigenesis, consistent with a causal role. MCPyV infection is widespread in humans, with over 80% seropositivity in individuals over 50 years of age. This virus has a typical polyomavirus ~5000 base pair genome encoding capsid proteins and LT and ST, both of which can induce tumors in transgenic mice.[257,267,290] Ongoing expression of the viral oncogenes is required for growth of MCC cells in culture.[146]

As is the case for other polyomaviruses, MCPyV LT binds and inactivates RB, leading to the transcription of E2F-regulated genes.[143] As a consequence of viral DNA integration, MCC tumors generally express a truncated form of LT preserving the amino-terminal J domain and RB binding site, but inactivating the replication functions of LT.[107,256] ST is also a major MCPyV oncogene that, as described above, indirectly neutralizes the p53 pathway by inducing mdm2. The ST-MYCL complex also drives transcription of LSD1, a demethylase that inhibits ATOH1-dependent transcription. Because ATOH1 is required for neuroendocrine cell differentiation, ATOH-1 inhibition contributes to maintaining cells

in a stemlike state.[138]

Twenty percent of MCCs lack MCPyV DNA and display a mutational signature consistent with UV exposure, while this signature is not present in virus-associated MCC.[120,137] Furthermore, nonviral MCCs carry many mutations and chromosomal rearrangements, whereas virus-associated tumors contain few mutations and typically have a normal karyotype.[269,270] Nevertheless, many of the same cellular processes and pathways are altered in both virus-associated and nonviral MCC. For example, in virus-associated MCC, RB and p53 are inactivated by LT and ST, respectively, whereas UV-associated MCCs frequently carry mutations that inactivate RB and p53.[122,137,143] Similarly, in tumors driven by MCPyV, ST binds and activates MYCL, while MYCL is often overexpressed in nonviral MCC.[219,270]

Epstein-Barr Virus

Epstein-Barr virus, a γ-herpesvirus with a large (~170 000 base pair) double-stranded DNA genome, can replicate in both B lymphocytes and epithelial cells, a cell tropism that is reflected in the cancers associated with EBV. More than 90% of the adult human population is seropositive for EBV. Although many EBV infections are subclinical, EBV causes infectious mononucleosis, a usually mild disease caused by polyclonal expansion of B lymphocytes.

EBV is associated with several human cancers including Burkitt lymphoma (BL), classic Hodgkin lymphoma and other B cell tumors, nasopharyngeal carcinoma, and gastric carcinoma. EBV is latent in cancer cells with the viral genome tethered to the host chromosomes and only a small subset of viral genes is expressed. There are four types of EBV latency defined by unique viral gene expression profiles.[300] In latency 0, the only viral genes expressed are the ncRNAs EBER-1 and EBER-2 and other transcripts named BARTs that include viral miRNAs and other ncRNAs. In latency I, EBNA1 is expressed in addition to these ncRNAs. Latency II is characterized by the additional expression of LMP1, LMP2A, and LMP2B. Finally, in latency III, viral gene expression is expanded to include EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP. Each type of EBV-associated cancer displays a characteristic latency pattern.

Burkitt Lymphoma. BL is an aggressive B cell lymphoma that occurs in endemic, sporadic, and immunodeficiency-associated forms.[28,38] EBV DNA is found in about 95% of the endemic BL and in about 30% of sporadic and immunodeficiency-associated BL.[300] Endemic BL is a common pediatric tumor in sub-Saharan Africa, and its development usually requires cooperation between EBV and malaria. All BLs carry a fusion of c-*myc* and an immunoglobulin (Ig) gene resulting in overexpression of Myc, which drives cell proliferation and induces apoptosis. EBV blocks Myc-induced apoptosis, thus allowing the survival of cells carrying the Ig-*myc* translocation.[165] In nonviral BL, apoptosis is abrogated by loss-of-function mutations in the p53 pathway or in downstream apoptosis effectors (reviewed by Ref.[282]).

EBV-positive BL displays latency I with EBNA1 being the only viral protein expressed. Inhibition of EBNA1 function in BL cell lines induces apoptosis, showing that ongoing action of EBNA1 is required for the survival of these cells.[165] BL cells also express EBER1 and EBER2 and viral miRNAs. During the establishment of latency, other early viral proteins including EBNA2, EBNA3A, and EBNA3C are transiently expressed. However, the combination of Myc overexpression and EBNA2 induces apoptosis.[163,217] Apoptosis is blocked by ENBA3A and EBNA3C, which silence the proapoptotic BIM gene. EBNA2 is usually then epigenetically silenced and latency I established. However, in a subset of BL, EBNA2 is deleted, causing repression of additional EBV genes including *BHRF1*, the viral homolog of Bcl-2, thereby blocking Myc-induced cell death.[164]

Classic Hodgkin lymphoma. EBV is associated with 10%-75% of classic Hodgkin lymphoma (CHL) cases, depending on the disease subtype.[169,300] EBV is in latency II, and the cells express EBNA1, LMP1, LMP2, and EBER. CHL cells have aberrantly rearranged Ig genes that trigger cell death, but LMP1 and LMP2A appear to block death by activating the NFkB pathway.[39,41]

Nasopharyngeal carcinoma (NPC) and *gastric carcinoma* are other EBV-associated cancers that exhibit latency II. Over 95% of NPC cases harbor EBV DNA, as do 10% of gastric cancers.[6,20,43,218] Gastric cancers can be grouped into distinct subtypes based the number and types of mutations.[43] EBV-associated gastric cancers form a distinct molecular subtype characterized by DNA hypermethylation, mutations in the PI3K pathway, and amplification of *JAK2* and *CD274*. These tumors rarely carry p53 mutations, while p53 is frequently mutated in non-EBV associated cancers. This unique genetic signature suggests a driver role for EBV in a subset of gastric cancers. *NPC* predominantly occurs in certain areas in Southeast Asia.[252] The basis for this striking geographical distribution is not known, but environmental factors such as a diet rich in fermented and preserved foods may play a role.[309] In addition, host HLA genotype affects the risk of EBV-induced NPC.[185]

EBV also causes lymphoproliferative disease in individuals with immunodeficiency, including central nervous system lymphomas in patients with acquired immunodeficiency syndrome (AIDS) and following bone marrow transplantation. Adoptive immunotherapy with EBV-specific donor T cells can control EBV-related lymphomas in bone marrow transplant recipients, demonstrating the utility of targetting viral tumor antigens in immunotherapy.[144]

Kaposi Sarcoma–Associated Herpesvirus

Kaposi sarcoma–associated herpesvirus (KSHV) is another oncogenic γ-herpesvirus, with a ~165 000 base pair double-stranded DNA genome first detected in KS lesions.[53] The KSHV genome contains numerous genes homologous to cellular genes, which were presumably acquired by the viral genome in relatively recent evolutionary times. As described in an earlier section, many of these genes play key roles in cell growth control. KSHV infects multiple cell types and is usually latent in endothelial cells and B lymphocytes, where it expresses a limited number of viral genes including LANA, v-cyclin, v-FLIP, kaposins, v-IRF3, and viral miRNAs.[132,174] In addition to a causal role in KS, the virus has also been linked to primary effusion lymphoma (PEL), multicentric Castleman disease, and some diffuse large B-cell lymphomas.[49,211,265] The pathogenesis of KSHV-associated cancer is complex and poorly understood.[203]

Kaposi sarcoma. KS is a systemic disease affecting multiple cell types and organs including the skin, with a prominent vascular endothelial component.[5] Unlike many other tumors, KS lesions are not monoclonal and display features consistent with paracrine signaling, and cells derived from these lesions often do not display properties of transformed cells. KS is caused by KSHV infection, often in the setting of immunosuppression.[53] While all tumor cells harbor KSHV, the viral gene expression pattern varies in different cases, with some expressing only

latent genes while others display a lytic gene expression pattern.[86,87] Once a relatively rare tumor, the incidence of an aggressive form of KS increased greatly during the AIDS epidemic, but the incidence of AIDS-associated KS has dramatically decreased since the introduction of effective treatments for HIV infection.[25,102] In addition, the appearance of new KS lesions in AIDS patients is suppressed by treatment with the antiherpesviral drug, ganciclovir, which targets herpesvirus DNA polymerase.[48]

Primary effusion lymphoma. PEL occurs in AIDS patients, and all cases of PEL contain the KSHV genome.[7,204] These cancers overexpress *c-myc* due to the action of KSHV LANA. In addition, the PI3K, mTOR, and MAPK pathways appear critical for PEL survival.[23,36,189,259] PELs rarely contain activating PI3K mutations or loss-of-function mutations in PTEN, a negative regulator of PI3K, suggesting that viral proteins mediate the activation of these pathways. In fact, two KSHV proteins, K1 and vGPCR, have been reported to activate the PI3K pathway.[9,264,283]

KSHV is also associated with *multicentric Castleman disease (MCD)*, a polyclonal lymphoid malignancy and *KSHV-positive diffuse large B-cell lymphoma*, rare lymphoid malignancies associated with HIV infection and other forms of immunosuppression.[211,265] The mechanisms of carcinogenesis in these diseases is poorly understood, but recently progress in understanding MCD has been reviewed.[47]

Hepatitis B Virus

Chronic infection by HBV and HCV leads to the development of ~80% of HCC (Fig. 11.8).[226] HBV is a bloodborne pathogen that infects approximately one-third of people worldwide and causes acute and chronic hepatitis, as well as the majority of HCC.[18] HBV contains a small, partially double-stranded DNA genome and undergoes a complicated replication cycle involving RNA replicative intermediates.[275] In some HBV-infected individuals (particularly those infected as infants), infection becomes persistent. Persistent infection can lead to chronic liver damage (chronic active hepatitis [CAH]), which has a significant likelihood of progressing to HCC. Individuals coinfected with HBV and hepatitis delta virus, a satellite virus with an RNA genome, are at increased risk of developing HCC.[235] More than one billion people are vaccinated against HBV, representing a major public health triumph.

There are three major HCC subtypes.[44] HBV is most often associated with cluster 1 tumors, which are characterized by the overexpression of *MKI67*, *MYBL2*, and other cell cycle markers; a low frequency of *CDKN2A* silencing; and p53 mutations but not *hTERT* promoter mutations or overexpression. In addition, HBV-positive HCCs tend to have a mutational signature associated with exposure to the carcinogenic fungal natural product, aflatoxin. HBV-associated HCC frequently has integrated HBV genomes. In some cases, integration occurs near genes that are upregulated including *CCND1*, *CCNE*, *GLI2*, and *hTERT*, which are linked to cell cycle progression.[44,277,314]

Unlike the other DNA tumor viruses, HBV does not contain strong oncogenes, and its precise role in the development of HCC is unclear. However, HBV vaccination reduces the incidence of HCC, providing definitive evidence that HBV plays a causal role in this cancer (Fig. 11.11).[52,230,310] In addition, antiviral therapy can reduce HCC risk in patients with chronic HBV infection, especially if treatment is initiated early in the disease.[304] Three broad, nonexclusive hypotheses have been proposed to explain the role of HBV in HCC. First, HBV acts as an indirect mutagen in which HBV replication causes inflammation, cell death, and repeated cycles of hepatocyte proliferation and mutagenesis during regeneration resulting in the accumulation of cellular mutations that lead to cancer formation.[235] Second, mutagenesis or gene activation mediated by HBV DNA integration into the cell genome drives HCC.[105,284,311] Finally, it is possible that a viral oncogene contributes to tumorigenesis. For example, the HBV *X* gene has been implicated in cell transformation and tumorigenesis in transgenic mice.[16,260] *X* affects cellular gene expression and may interfere with p53 function.[106]

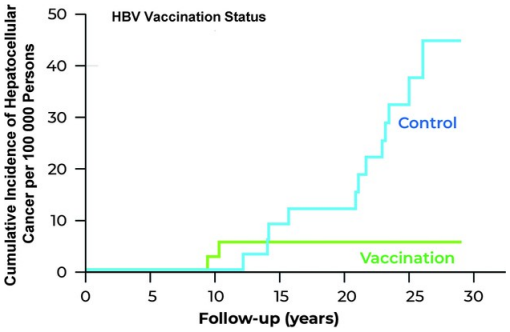

FIGURE 11.11 **HBV vaccination can prevent cancer**. Graph shows the cumulative incidence of hepatocellular carcinoma in HBV vaccinated and unvaccinated subjects as a function of years of follow-up. (Based on data published by Qu C, Chen T, Fan C, et al. Efficacy of neonatal HBV vaccination on liver cancer and other liver diseases over 30-year follow-up of the Qidong hepatitis B intervention study: a cluster randomized controlled trial. *PLoS Med*. 2014;11(12):e1001774. From DiMaio D, Emu B, Goodman AL, et al. Cancer microbiology. *J Natl Cancer Inst*. 2022;114(5):651-663. Reproduced by permission of Oxford University Press.)

Hepatitis C Virus

HCV belongs to the *Flaviviridae* family and has an ~9600-base single-stranded, positive-sense RNA genome.[62] HCV causes viral hepatitis and HCC and is also associated with non-Hodgkin B-cell lymphoma.[207,242] HCV is transmitted by blood, sexual activity, and intravenous drug use. Many HCV infections are inapparent and not diagnosed until late in the disease. In most populations, a few percent of people are seropositive for HCV, but rates of HCV positivity in Egypt approach 40% in some demographic groups because the virus was inadvertently transmitted during other medical interventions.[108] Unlike the situation with HBV, where a relatively small proportion of infections become chronic, HCV usually results in chronic infections and cirrhosis. In fact, HCV is the most common reason for liver transplantation in the Western world. HBV/HCV coinfections confer elevated risk of HCC development.

HCV lacks strong oncogenes, but viral structural genes have transforming or tumorigenic activity in various assays, in part by inducing genomic

instability.[182,200,262] The carcinogenic potential of HCV is thought to derive primarily from its cytolytic activity in hepatocytes and the resulting inflammation, cell regeneration, and mutagenesis. HCV-positive tumors are associated with *CDKN2A* promoter silencing and *hTERT* promoter mutation.[44] Direct acting antiviral drugs that target HCV replication proteins such as proteases and polymerase can cure HCV infections in nearly all patients infected with common virus strains, and successful treatment reduces the risk of HCC.[151,158]

Human T-Cell Leukemia Virus Type 1

Human T-cell leukemia virus type 1 (HTLV-1), the first discovered human retrovirus, infects ~20 million people worldwide, with high endemic areas in Latin America, the Caribbean, Africa, and Japan.[227,307] HTLV-1 is transmitted by infected blood and other body fluids. Breast-feeding is a primary mode of HTLV-1 transmission, and refraining from this practice appears to reduce the incidence of vertical HTLV-1 transmission.[31] HTLV-1 infection is associated with adult T-cell leukemia/lymphoma (ATL) and some inflammatory diseases.[115] ATL typically arises several decades after HTLV-1 infection and is rapidly progressive, and it occurs in only a small fraction of infected individuals, suggesting the importance of additional, possibly nonviral events such as the accumulation of cellular mutations. Host genetics can also influence the risk of developing ATL.

HTLV-1 contains several accessory genes in addition to *gag*, *pol*, and *env*. During normal infection, the multifunctional accessory protein Tax regulates HTLV-1 gene expression from the viral LTR by interacting with cellular transcription factors.[75] Tax can induce lymphoid and mesenchymal tumors in transgenic mice and appears to play a role in transforming T lymphocytes by affecting transcription of cellular genes, inducing genomic instability, and influencing a variety of other cellular processes.[12,115,127,140] HTLV-1 also expresses a DNA binding protein, HBZ (a B-zip factor) that can induce lymphomas in animals.[244] HBZ but not Tax is invariably expressed in ATL cells. HBZ and Tax may cooperate in tumorigenesis, with Tax playing an initiating role and HBZ playing a role in tumor maintenance.[196]

Retroviral Oncogenesis in Gene Therapy Trials

Because retroviruses can stably deliver genes into cell DNA, they have been used as gene transfer vectors to correct inherited genetic disorders such as immunodeficiency syndromes.[208] One early trial studied treated boys with X-linked severe combined immunodeficiency (X-SCID), which is caused by mutations in the IL2 receptor γ gene (*IL2RG*). In this trial, hematopoietic progenitor cells were isolated from X-SCID patients, infected *ex vivo* with an MuLV-based vector lacking viral genes but expressing *IL2RG*, and then reinfused.[135] This treatment corrected the SCID defect, but a majority of the subjects subsequently developed T-cell acute lymphoblastic leukemia. Molecular analysis of the tumors revealed that the retroviral vector had integrated near the *LMO2* or the *CCND2* protooncogene, resulting in increased oncogene transcription.[134] Thus, the gene therapy vector appears to cause insertional activation of cellular protooncogenes. Similar outcomes involving other protooncogenes also occurred in trials treating other immune disorders.[34,173,272] Improved viral vectors have been developed that appear to reduce the incidence of these adverse outcomes (eg, see Ref.[145]).

Human Immunodeficiency Virus

HIV is a lentivirus that, like other retroviruses, undergoes reverse transcription and stable integration into the host cell genome. HIV infects $CD4^+$ T lymphocytes and can persist in a latent state for many years. In the absence of treatment, HIV infection eventually leads to profound T-cell depletion and severe immunosuppression, resulting in AIDS and fatal opportunistic infections. Worldwide, more that 40 million people have succumbed to AIDS, and HIV currently infects more than 30 million people. HIV is a bloodborne pathogen, transmitted by blood transfusions, needle sharing, and unprotected sexual contact. Public health measures such as blood donor screening has reduced certain modes of transmission.

HIV infection confers substantial excess risk for many cancers, and cancer prognosis in HIV-infected individuals is often worse than for people without HIV.[67,68] In North America, cancer accounts for up to 20% of deaths in HIV-positive individuals. Cervical cancer, KS, and non-Hodgkin lymphoma in the context of an active HIV infection establish the diagnosis of AIDS and are known as AIDS-defining cancers (ADCs). Interestingly, ADC are caused by tumor viruses—HPV, KSHV, and EBV. It is thought that HIV-induced immunosuppression allows unrestrained tumor virus replication and the resulting carcinogenic stimulus.[109]

Combination antiretroviral therapy that inhibits HIV enzymes required for virus replication—reverse transcriptase, integrase, and protease—suppresses HIV replication, and now millions of people are successfully treated (but not cured) for their HIV infection. Antiretroviral therapy substantially reduces the incidence of ADCs, demonstrating that antiviral therapy can prevent cancer.[236,255] Despite this decrease, people living with treated HIV remain at increased risk for ADCs compared to uninfected people. They are also at increased risk of other malignancies known as non-AIDS-defining cancers, which now account for two-thirds of cancers in HIV patients, with lung cancer being the most common.[103,258] It is not known if this elevated risk is due to residual immune dysfunction despite good HIV control or to some other factor.[237]

Human Endogenous Retroviruses

About 8% of the human genome is the remnant of ancient retrovirus infections of the germline. There is limited understanding of the role, if any, of human endogenous retroviruses (HERVs) in cancer. Most HERVs are epigenetically silenced, but they are often derepressed in cancer and might stimulate tumorigenesis by expressing carcinogenic gene products or by influencing the expression of cellular genes.[13] Conversely, agents that reverse repression of HERVs in cancer cells may stimulate a protective immune response.[312] These divergent possibilities suggest that HERVs may play a complex role in cancer formation that deserves further attention.

Future Perspectives

Tumor virology has provided important insight into mechanisms of carcinogenesis and led to powerful new approaches to prevent cancer, but many key questions remain, some with immediate public health consequences. How do we consolidate and extend the successes already

achieved in preventing and treating cancers caused by viruses, are there additional viruses that cause human cancers, and how do we prepare ourselves against emerging tumor viruses?

Efforts must be redoubled to provide better coverage with existing and future HPV and HBV vaccines, particularly in LMIC. Prophylactic vaccines are also needed for HCV, EBV, KSHV, and HIV. Recent advances in structural biology and vaccine design (including viral vaccine vectors and mRNA vaccines) and better understanding of conformation-specific antigens generate hope that effective vaccines against these viruses are attainable. Because of the relatively small number of people affected by MCC and ATL and the cost of vaccine development, testing, and distribution, vaccines against MCPyV and HTLV-1 are less likely to be developed. HIV, HBV, and HCV antivirals reduce cancer incidence, so the development and distribution of drugs against these viruses should continue apace. However, because of the expense of some of curative regimens, prophylactic vaccines seem the best long-term solution.

Have we discovered all of the human tumor viruses and do known tumor viruses cause additional cancers? Reports have linked cancers to viruses other than the known carcinogenic human viruses. Several studies have reported human cytomegalovirus (hCMV) DNA sequences in glioblastomas.[92] However, neither hCMV DNA or mRNA has been detected in tumor sequencing projects. Similarly, JC polyomavirus (JCPyV) DNA sequences have been reported in colorectal preneoplastic lesions and cancers but are not present in tumor sequence databases. It is unclear if hCMV and JCPyV are just passengers or are contributing to these neoplasias.

There appears to be a link between HPV and BK polyomavirus (BKPyV) and a small fraction of urinary tract tumors. BKPyV DNA sequences have been detected in urinary tract tumors associated with post renal transplant immunosuppressive therapy, and HPV and BKPyV genomes were found integrated in 19/268 sequenced bladder cancers. These tumors expressed the HPV E7 and E6 or the BKPyV LT oncogenes.[46] While half of the virus-negative tumors harbored p53 mutations, all of the virus-positive tumors expressed wild-type p53. Similarly, mutations in the RB-E2F pathway were found in virus-negative, but not virus-positive tumors. This pattern suggests that HPV and BKPyV drive a small subset of urinary track carcinomas.[268]

Although available cancer sequencing data suggest that unrecognized tumor viruses are unlikely to cause a substantial number of human cancers, it is possible that viruses cause human cancer via a hit-and-run mechanism. If this occurs, the number of cancers triggered by viruses may be higher (possibly even substantially higher) than is currently appreciated. Epidemiological data may provide initial clues of such associations, but novel laboratory- and computation-based methods will be required to explore this issue definitively. Cancers whose incidence is decreased by antiviral strategies are prime candidates for detailed investigation into possible viral causes.

A final concern is emerging tumor virus infections. It is possible that some viruses with cancer-causing potential or immunosuppressive properties exist but have not yet entered the human population from their natural animal hosts. If such an event occurs, it probably will not be immediately recognizable as a cause of cancer, because the time-course of virus-induced tumorigenesis in humans typically lasts decades. Therefore, the emergence of a new carcinogenic virus might not be recognized until long after the initial introduction of the virus into humans, providing years for the virus to become established and widely disseminated in humans. We must fortify ourselves against this possibility by continuing to conduct studies of basic virology, biochemistry and structural biology, viral immunology, and related areas so that we have the knowledge to rapidly counter newly emerging viral disease. We must also build and maintain strong surveillance programs to rapidly identify new viruses in the human population so that we can combat them before they spread widely.

Since tumor viruses were discovered in 1908, tumor virology has progressed through several phases. Descriptive studies evolved into mechanistic ones, tools developed to study tumor viruses were deployed to revolutionize all of biology, and hints of associations between viruses and cancer led to clear understanding of the carcinogenic activities of specific viruses in humans and ultimately to powerful new interventions to prevent certain cancers. Additional studies of viruses and cancer will continue to have important impact on biomedical science and public health.

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REFERENCES

1. An P, Saenz Robles MT, Pipas JM. Large T antigens of polyomaviruses: amazing molecular machines. Annu Rev Microbiol. 2012;66:213-236.

2. Anaman-Torgbor J, Angmorterh SK, Dordunoo D, Ofori EK. Cervical cancer screening behaviours and challenges: a sub-Saharan Africa perspective. Pan Afr Med J. 2020;36:97.

3. Anderton E, Yee J, Smith P, Crook T, White RE, Allday MJ. Two Epstein-Barr virus (EBV) oncoproteins cooperate to repress expression of the proapoptotic tumour-suppressor Bim: clues to the pathogenesis of Burkitt's lymphoma. Oncogene. 2008;27(4):421-433.

4. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med. 2010;363(1):24-35.

5. Antman K, Chang Y. Kaposi's sarcoma. N Engl J Med. 2000;342(14):1027-1038.

6. Argirion I, Zarins KR, Ruterbusch JJ, et al. Increasing incidence of Epstein-Barr virus-related nasopharyngeal carcinoma in the United States. Cancer. 2020;126(1):121-130.

7. Arvanitakis L, Mesri EA, Nador RG, et al. Establishment and characterization of a primary effusion (body cavity-based) lymphoma cell line (BC-3) harboring kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) in the absence of Epstein-Barr virus. Blood. 1996;88(7):2648-2654.

8. Bais C, Santomasso B, Coso O, et al. G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. Nature. 1998;391(6662):86-89.

9. Bais C, Van Geelen A, Eroles P, et al. Kaposi's sarcoma associated herpesvirus G protein-coupled receptor immortalizes human endothelial cells by activation of the VEGF receptor-2/KDR. Cancer Cell. 2003;3(2):131-143.

10. Ballestas ME, Chatis PA, Kaye KM. Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. Science. 1999;284(5414):641-644.

11. Ballon G, Chen K, Perez R, Tam W, Cesarman E. Kaposi sarcoma herpesvirus (KSHV) vFLIP oncoprotein induces B cell transdifferentiation and tumorigenesis in mice. J Clin Invest. 2011;121(3):1141-1153.

12. Bangham CR, Ratner L. How does HTLV-1 cause adult T-cell leukaemia/lymphoma (ATL)? Curr Opin Virol. 2015;14:93-100.

13. Bannert N, Hofmann H, Block A, Hohn O. HERVs new role in cancer: from accused perpetrators to cheerful protectors. Front Microbiol. 2018;9:178.

14. Barbera AJ, Chodaparambil JV, Kelley-Clarke B, et al. The nucleosomal surface as a docking station for Kaposi's sarcoma herpesvirus LANA. Science. 2006;311(5762):856-861.

15. Baresova P, Musilova J, Pitha PM, Lubyova B. p53 tumor suppressor protein stability and transcriptional activity are targeted by Kaposi's sarcoma-associated herpesvirus-encoded viral interferon regulatory factor 3. Mol Cell Biol. 2014;34(3):386-399.

16. Basile JR, Zacny V, Munger K. The cytokines tumor necrosis factor-alpha (TNF-alpha) and TNF-related apoptosis-inducing ligand differentially modulate proliferation and apoptotic pathways in human keratinocytes expressing the human papillomavirus-16 E7 oncoprotein. J Biol Chem. 2001;276(25):22522-22528.

17. Basukala O, Banks L. The not-so-good, the bad and the ugly: HPV E5, E6 and E7 oncoproteins in the orchestration of carcinogenesis. Viruses. 2021;13(10):1892.

18. Beasley RP, Hwang LY, Lin CC, Chien CS. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan. Lancet. 1981;2(8256):1129-1133.

19. Becker JC, Stang A, DeCaprio JA, et al. Merkel cell carcinoma. Nat Rev Dis Primers. 2017;3:17077.

20. Bell AI, Groves K, Kelly GL, et al. Analysis of Epstein-Barr virus latent gene expression in endemic Burkitt's lymphoma and nasopharyngeal carcinoma tumour cells by using quantitative real-time PCR assays. J Gen Virol. 2006;87(Pt 10):2885-2890.

21. Bellacosa A, Testa JR, Staal SP, Tsichlis PN. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. Science. 1991;254(5029):274-277.

22. Ben David Y, Prideaux VR, Chow V, Benchimol S, Bernstein A. Inactivation of the p53 oncogene by internal deletion or retroviral integration in erythroleukemic cell lines induced by Friend leukemia virus. Oncogene. 1988;3(2):179-185.

23. Bhatt AP, Bhende PM, Sin SH, Roy D, Dittmer DP, Damania B. Dual inhibition of PI3K and mTOR inhibits autocrine and paracrine proliferative loops in PI3K/Akt/mTOR-addicted lymphomas. Blood. 2010;115(22):4455-4463.

24. Biedenkapp H, Borgmeyer U, Sippel AE, Klempnauer KH. Viral myb oncogene encodes a sequence-specific DNA-binding activity. Nature. 1988;335(6193):835-837.

25. Biggar RJ, Chaturvedi AK, Goedert JJ, Engels EA; HIV/AIDS Cancer Match Study. AIDS-related cancer and severity of immunosuppression in persons with AIDS. J Natl Cancer Inst. 2007;99(12):962-972.

26. Bishop JM. Viral oncogenes. Cell. 1985;42(1):23-38.

27. Blenis J. Signal transduction via the MAP kinases: proceed at your own RSK. Proc Natl Acad Sci U S A. 1993;90(13):5889-5892.

28. Blum KA, Lozanski G, Byrd JC. Adult Burkitt leukemia and lymphoma. Blood. 2004;104(10):3009-3020.

29. Bohmann D, Bos TJ, Admon A, Nishimura T, Vogt PK, Tjian R. Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science. 1987;238(4832):1386-1392.

30. Bollag G, McCormick F. Regulators and effectors of ras proteins. Annu Rev Cell Biol. 1991;7:601-632.

31. Boostani R, Sadeghi R, Sabouri A, Ghabeli-Juibary A. Human T-lymphotropic virus type I and breastfeeding; systematic review and meta-analysis of the literature. Iran J Neurol. 2018;17(4):174-179.

32. Bos TJ, Monteclaro FS, Mitsunobu F, et al. Efficient transformation of chicken embryo fibroblasts by c-Jun requires structural modification in coding and noncoding sequences. Genes Dev. 1990;4(10):1677-1687.

33. Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W, zur Hausen H. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J. 1984;3(5):1151-1157.

34. Boztug K, Schmidt M, Schwarzer A, et al. Stem-cell gene therapy for the Wiskott-Aldrich syndrome. N Engl J Med. 2010;363(20):1918-1927.

35. Brugge JS, Erikson RL. Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature. 1977;269(5626):346-348.

36. Bubman D, Guasparri I, Cesarman E. Deregulation of c-Myc in primary effusion lymphoma by Kaposi's sarcoma herpesvirus latency-associated nuclear antigen. Oncogene. 2007;26(34):4979-4986.

37. Bugter JM, Fenderico N, Maurice MM. Mutations and mechanisms of WNT pathway tumour suppressors in cancer. Nat Rev Cancer. 2021;21(1):5-21.

38. Burkitt D. A children's cancer dependent on climatic factors. Nature. 1962;194:232-234.

39. Cahir-McFarland ED, Carter K, Rosenwald A, et al. Role of NF-kappa B in cell survival and transcription of latent membrane protein 1-expressing or Epstein-Barr virus latency III-infected cells. J Virol. 2004;78(8):4108-4119.

40. Cahir-McFarland ED, Davidson DM, Schauer SL, Duong J, Kieff E. NF-kappa B inhibition causes spontaneous apoptosis in Epstein-Barr virus-transformed lymphoblastoid cells. Proc Natl Acad Sci U S A. 2000;97(11):6055-6060.

41. Caldwell RG, Wilson JB, Anderson SJ, Longnecker R. Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. Immunity. 1998;9(3):405-411.

42. Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015;517(7536):576-582.

43. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014;513(7517):202-209.

44. Cancer Genome Atlas Research Network. Electronic address wbe, Cancer Genome Atlas Research Network. Comprehensive and integrative genomic characterization of hepatocellular carcinoma. Cell. 2017;169(7):1327-1341 e23.

45. Cannon ML, Cesarman E. The KSHV G protein-coupled receptor signals via multiple pathways to induce transcription factor activation in primary effusion lymphoma cells. Oncogene. 2004;23(2):514-523.

46. Cantalupo PG, Katz JP, Pipas JM. Viral sequences in human cancer. Virology. 2018;513:208-216.

47. Carbone A, Borok M, Damania B, et al. Castleman disease. Nat Rev Dis Primers. 2021;7(1):84.

48. Casper C. New approaches to the treatment of human herpesvirus 8-associated disease. Rev Med Virol. 2008;18(5):321-329.

49. Cesarman E, Chang Y, Moore PS, Said JW, Knowles DM. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N Engl J Med. 1995;332(18):1186-1191.

50. Cesarman E, Nador RG, Bai F, et al. Kaposi's sarcoma-associated herpesvirus contains G protein-coupled receptor and cyclin D homologs which are expressed in Kaposi's sarcoma and malignant lymphoma. J Virol. 1996;70(11):8218-8223.

51. Chang HW, Aoki M, Fruman D, et al. Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase. Science. 1997;276(5320):1848-1850.

52. Chang MH, You SL, Chen CJ, et al. Decreased incidence of hepatocellular carcinoma in hepatitis B vaccinees: a 20-year follow-up study. J Natl Cancer Inst. 2009;101(19):1348-1355.

53. Chang Y, Cesarman E, Pessin MS, et al. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science. 1994;266(5192):1865-1869.

54. Chang Y, Moore PS, Talbot SJ, et al. Cyclin encoded by KS herpesvirus. Nature. 1996;382(6590):410.

55. Chatterjee M, Osborne J, Bestetti G, Chang Y, Moore PS. Viral IL-6-induced cell proliferation and immune evasion of interferon activity. Science. 2002;298(5597):1432-1435.

56. Chaudhary PM, Jasmin A, Eby MT, Hood L. Modulation of the NF-kappa B pathway by virally encoded death effector domains-containing proteins. Oncogene. 1999;18(42):5738-5746.

57. Cheng EH, Nicholas J, Bellows DS, et al. A Bcl-2 homolog encoded by Kaposi sarcoma-associated virus, human herpesvirus 8, inhibits apoptosis but does not heterodimerize with Bax or Bak. Proc Natl Acad Sci U S A. 1997;94(2):690-694.

58. Cheng J, Park DE, Berrios C, et al. Merkel cell polyomavirus recruits MYCL to the EP400 complex to promote oncogenesis. PLoS Pathog. 2017;13(10):e1006668.

59. Cheng J, Rozenblatt-Rosen O, Paulson KG, Nghiem P, DeCaprio JA. Merkel cell polyomavirus large T antigen has growth-promoting and inhibitory activities. J Virol. 2013;87(11):6118-6126.

60. Chiu IM, Reddy EP, Givol D, Robbins KC, Tronick SR, Aaronson SA. Nucleotide sequence analysis identifies the human c-sis proto-oncogene as a structural gene for platelet-derived growth factor. Cell. 1984;37(1):123-129.

61. Chiu R, Boyle WJ, Meek J, Smeal T, Hunter T, Karin M. The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell. 1988;54(4):541-552.

62. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science. 1989;244(4902):359-362.

63. Christensen ND, Hopfl R, DiAngelo SL, et al. Assembled baculovirus-expressed human papillomavirus type 11 L1 capsid protein virus-like particles are recognized by neutralizing monoclonal antibodies and induce high titres of neutralizing antibodies. J Gen Virol. 1994;75 (Pt 9):2271-2276.

64. Chugh P, Matta H, Schamus S, et al. Constitutive NF-kappaB activation, normal Fas-induced apoptosis, and increased incidence of

lymphoma in human herpes virus 8 K13 transgenic mice. Proc Natl Acad Sci U S A. 2005;102(36):12885-12890.

65. Cmarik J, Ruscetti S. Friend spleen focus-forming virus activates the tyrosine kinase sf-Stk and the transcription factor PU.1 to cause a multi-stage erythroleukemia in mice. Viruses. 2010;2(10):2235-2257.

66. Coffin JM, Varmus HE, Bishop JM, et al. Proposal for naming host cell-derived inserts in retrovirus genomes. J Virol. 1981;40(3):953-957.

67. Coghill AE, Pfeiffer RM, Shiels MS, Engels EA. Excess mortality among HIV-infected individuals with cancer in the United States. Cancer Epidemiol Biomarkers Prev. 2017;26(7):1027-1033.

68. Coghill AE, Suneja G, Rositch AF, Shiels MS, Engels EA. HIV infection, cancer treatment regimens, and cancer outcomes among elderly adults in the United States. JAMA Oncol. 2019;5(9):e191742.

69. Cohen JI, Wang F, Mannick J, Kieff E. Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. Proc Natl Acad Sci U S A. 1989;86(23):9558-9562.

70. Collett MS, Purchio AF, Erikson RL. Avian sarcoma virus-transforming protein, pp60src shows protein kinase activity specific for tyrosine. Nature. 1980;285(5761):167-169.

71. Copeland NG, Jenkins NA. Myeloid leukemia: disease genes and mouse models. Prog Exp Tumor Res. 1999;35:53-63.

72. Cotter MA II, Robertson ES. The latency-associated nuclear antigen tethers the Kaposi's sarcoma-associated herpesvirus genome to host chromosomes in body cavity-based lymphoma cells. Virology. 1999;264(2):254-264.

73. Courtneidge SA. Activation of the pp60c-src kinase by middle T antigen binding or by dephosphorylation. EMBO J. 1985;4(6):1471-1477.

74. Courtneidge SA, Smith AE. Polyoma virus transforming protein associates with the product of the c-src cellular gene. Nature. 1983;303(5916):435-439.

75. Currer R, Van Duyne R, Jaworski E, et al. HTLV tax: a fascinating multifunctional co-regulator of viral and cellular pathways. Front Microbiol. 2012;3:406.

76. Damm K, Thompson CC, Evans RM. Protein encoded by v-erbA functions as a thyroid-hormone receptor antagonist. Nature. 1989;339(6226):593-597.

77. Danna K, Nathans D. Specific cleavage of simian virus 40 DNA by restriction endonuclease of Hemophilus influenzae. Proc Natl Acad Sci U S A. 1971;68(12):2913-2917.

78. Danna KJ, Nathans D. Bidirectional replication of Simian Virus 40 DNA. Proc Natl Acad Sci U S A. 1972;69(11):3097-3100.

79. Danna KJ, Sack GH Jr, Nathans D. Studies of simian virus 40 DNA. VII. A cleavage map of the SV40 genome. J Mol Biol. 1973;78(2):363-376.

80. Dawson CW, Tramountanis G, Eliopoulos AG, Young LS. Epstein-Barr virus latent membrane protein 1 (LMP1) activates the phosphatidylinositol 3-kinase/Akt pathway to promote cell survival and induce actin filament remodeling. J Biol Chem. 2003;278(6):3694-3704.

81. de Martel C, Georges D, Bray F, Ferlay J, Clifford GM. Global burden of cancer attributable to infections in 2018: a worldwide incidence analysis. Lancet Glob Health. 2020;8(2):e180-e190.

82. de Sauvage FJ, Hass PE, Spencer SD, et al. Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. Nature. 1994;369(6481):533-538.

83. DiMaio D. Viral miniproteins. Annu Rev Microbiol. 2014;68:21-43.

84. DiMaio D. Small size, big impact: how studies of small DNA tumour viruses revolutionized biology. Philos Trans R Soc Lond B Biol Sci. 2019;374(1773):20180300.

85. DiMaio D, Emu B, Goodman AL, Mothes M, Justice A. Cancer microbiology. J Natl Cancer Inst. 2022;114(5):651-663.

86. Dittmer D, Lagunoff M, Renne R, Staskus K, Haase A, Ganem D. A cluster of latently expressed genes in Kaposi's sarcoma-associated herpesvirus. J Virol. 1998;72(10):8309-8315.

87. Dittmer DP. Restricted Kaposi's sarcoma (KS) herpesvirus transcription in KS lesions from patients on successful antiretroviral therapy. MBio. 2011;2(6):e00138-11.

88. Downward J, Yarden Y, Mayes E, et al. Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature. 1984;307(5951):521-527.

89. Duesberg PH, Kawai S, Wang LH, Vogt PK, Murphy HM, Hanafusa H. RNA of replication-defective strains of Rous sarcoma virus. Proc Natl Acad Sci U S A. 1975;72(4):1569-1573.

90. Durst M, Dzarlieva-Petrusevska RT, Boukamp P, Fusenig NE, Gissmann L. Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. Oncogene. 1987;1(3):251-256.

91. Durst M, Gissmann L, Ikenberg H, zur Hausen H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc Natl Acad Sci U S A. 1983;80(12):3812-3815.

92. Dziurzynski K, Chang SM, Heimberger AB, et al. Consensus on the role of human cytomegalovirus in glioblastoma. Neuro Oncol. 2012;14(3):246-255.

93. Eckhart W, Hutchinson MA, Hunter T. An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. Cell. 1979;18(4):925-933.

94. Eddy BE, Borman GS, Grubbs GE, Young RD. Identification of the oncogenic substance in rhesus monkey kidney cell culture as simian virus 40. Virology. 1962;17:65-75.

95. Eichten A, Rud DS, Grace M, Piboonniyom SO, Zacny V, Munger K. Molecular pathways executing the “trophic sentinel” response in HPV-16 E7-expressing normal human diploid fibroblasts upon growth factor deprivation. Virology. 2004;319(1):81-93.

96. Eilers M, Eisenman RN. Myc’s broad reach. Genes Dev. 2008;22(20):2755-2766.

97. Eliopoulos AG, Gallagher NJ, Blake SM, Dawson CW, Young LS. Activation of the p38 mitogen-activated protein kinase pathway by Epstein-Barr virus-encoded latent membrane protein 1 coregulates interleukin-6 and interleukin-8 production. J Biol Chem. 1999;274(23):16085-16096.

98. Eliopoulos AG, Young LS. Activation of the cJun N-terminal kinase (JNK) pathway by the Epstein-Barr virus-encoded latent membrane protein 1 (LMP1). Oncogene. 1998;16(13):1731-1742.

99. Ellermann V, Bang O. Experimentelle Leukamie bei Huhnern. Zentralbl Bakteriol Parasitenkd Infectionskr Hyg Abt I. 1908;46:595-609.

100. Ellis RW, Defeo D, Shih TY, et al. The p21 src genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. Nature. 1981;292(5823):506-511.

101. Engels EA, Frisch M, Goedert JJ, Biggar RJ, Miller RW. Merkel cell carcinoma and HIV infection. Lancet. 2002;359(9305):497-498.

102. Engels EA, Pfeiffer RM, Goedert JJ, et al. Trends in cancer risk among people with AIDS in the United States 1980-2002. AIDS. 2006;20(12):1645-1654.

103. Engels EA, Yanik EL, Wheeler W, et al. Cancer-attributable mortality among people with treated human immunodeficiency virus infection in North America. Clin Infect Dis. 2017;65(4):636-643.

104. Fakhari FD, Jeong JH, Kanan Y, Dittmer DP. The latency-associated nuclear antigen of Kaposi sarcoma-associated herpesvirus induces B cell hyperplasia and lymphoma. J Clin Invest. 2006;116(3):735-742.

105. Feitelson MA, Lee J. Hepatitis B virus integration, fragile sites, and hepatocarcinogenesis. Cancer Lett. 2007;252(2):157-170.

106. Feitelson MA, Zhu M, Duan LX, London WT. Hepatitis B x antigen and p53 are associated in vitro and in liver tissues from patients with primary hepatocellular carcinoma. Oncogene. 1993;8(5):1109-1117.

107. Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science. 2008;319(5866):1096-1100.

108. Frank C, Mohamed MK, Strickland GT, et al. The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. Lancet. 2000;355(9207):887-891.

109. Frisch M, Biggar RJ, Engels EA, Goedert JJ; AIDS-Cancer Match Registry Study Group. Association of cancer with AIDS-related immunosuppression in adults. JAMA. 2001;285(13):1736-1745.

110. Fujimuro M, Wu FY, ApRhys C, et al. A novel viral mechanism for dysregulation of beta-catenin in Kaposi’s sarcoma-associated herpesvirus latency. Nat Med. 2003;9(3):300-306.

111. Fukuda M, Longnecker R. Epstein-Barr virus latent membrane protein 2A mediates transformation through constitutive activation of the Ras/PI3-K/Akt Pathway. J Virol. 2007;81(17):9299-9306.

112. Gaglia MM, Munger K. More than just oncogenes: mechanisms of tumorigenesis by human viruses. Curr Opin Virol. 2018;32:48-59.

113. Gao SJ, Boshoff C, Jayachandra S, Weiss RA, Chang Y, Moore PS. KSHV ORF K9 (vIRF) is an oncogene which inhibits the interferon signaling pathway. Oncogene. 1997;15(16):1979-1985.

114. Genther Williams SM, Disbrow GL, Schlegel R, Lee D, Threadgill DW, Lambert PF. Requirement of epidermal growth factor receptor for hyperplasia induced by E5, a high-risk human papillomavirus oncogene. Cancer Res. 2005;65(15):6534-6542.

115. Giam CZ, Jeang KT. HTLV-1 Tax and adult T-cell leukemia. Front Biosci. 2007;12:1496-1507.

116. Gillison ML, Chaturvedi AK, Lowy DR. HPV prophylactic vaccines and the potential prevention of noncervical cancers in both men and women. Cancer. 2008;113(10 Suppl):3036-3046.

117. Gilmore TD, Kalaitzidis D, Liang MC, Starczynowski DT. The c-Rel transcription factor and B-cell proliferation: a deal with the devil. Oncogene. 2004;23(13):2275-2286.

118. Girardi AJ, Sweet BH, Slotnick VB, Hilleman MR. Development of tumors in hamsters inoculated in the neonatal period with vacuolating virus, SV-40. Proc Soc Exp Biol Med. 1962;109:649-660.

119. Glykofrydes D, Niphuis H, Kuhn EM, et al. Herpesvirus saimiri vFLIP provides an antiapoptotic function but is not essential for viral replication, transformation, or pathogenicity. J Virol. 2000;74(24):11919-11927.

120. Goh G, Walradt T, Markarov V, et al. Mutational landscape of MCPyV-positive and MCPyV-negative Merkel cell carcinomas with implications for immunotherapy. Oncotarget. 2016;7(3):3403-3415.

121. Goldstein DJ, Andresson T, Sparkowski JJ, Schlegel R. The BPV-1 E5 protein, the 16 kDa membrane pore-forming protein and the PDGF receptor exist in a complex that is dependent on hydrophobic transmembrane interactions. EMBO J. 1992;11(13):4851-4859.

122. Gonzalez-Vela MDC, Curiel-Olmo S, Derdak S, et al. Shared oncogenic pathways implicated in both virus-positive and UV-induced merkel cell carcinomas. J Invest Dermatol. 2017;137(1):197-206.

123. Goodwin EC, DiMaio D. Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways. Proc Natl Acad Sci U S A. 2000;97(23):12513-12518.

124. Goodwin EC, Yang E, Lee CJ, Lee HW, DiMaio D, Hwang ES. Rapid induction of senescence in human cervical carcinoma cells. Proc Natl Acad Sci U S A. 2000;97(20):10978-10983.

125. Gordadze AV, Peng R, Tan J, et al. Notch1IC partially replaces EBNA2 function in B cells immortalized by Epstein-Barr virus. J Virol. 2001;75(13):5899-5912.

126. Grabowska AK, Riemer AB. The invisible enemy—how human papillomaviruses avoid recognition and clearance by the host immune system. Open Virol J. 2012;6:249-256.

127. Grassmann R, Aboud M, Jeang KT. Molecular mechanisms of cellular transformation by HTLV-1 Tax. Oncogene. 2005;24(39):5976-5985.

128. Greig KT, Carotta S, Nutt SL. Critical roles for c-Myb in hematopoietic progenitor cells. Semin Immunol. 2008;20(4):247-256.

129. Groffen J, Heisterkamp N, Shibuya M, Hanafusa H, Stephenson JR. Transforming genes of avian (v-fps) and mammalian (v-fes) retroviruses correspond to a common cellular locus. Virology. 1983;125(2):480-486.

130. Grossman SR, Johannsen E, Tong X, Yalamanchili R, Kieff E. The Epstein-Barr virus nuclear antigen 2 transactivator is directed to response elements by the J kappa recombination signal binding protein. Proc Natl Acad Sci U S A. 1994;91(16):7568-7572.

131. Grulich AE, Vajdic CM. The epidemiology of cancers in human immunodeficiency virus infection and after organ transplantation. Semin Oncol. 2015;42(2):247-257.

132. Grundhoff A, Ganem D. Inefficient establishment of KSHV latency suggests an additional role for continued lytic replication in Kaposi sarcoma pathogenesis. J Clin Invest. 2004;113(1):124-136.

133. Gwack Y, Hwang S, Byun H, et al. Kaposi's sarcoma-associated herpesvirus open reading frame 50 represses p53-induced transcriptional activity and apoptosis. J Virol. 2001;75(13):6245-6248.

134. Hacein-Bey-Abina S, Garrigue A, Wang GP, et al. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. J Clin Invest. 2008;118(9):3132-3142.

135. Hacein-Bey-Abina S, von Kalle C, Schmidt M, et al. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med. 2003;348(3):255-256.

136. Hammerschmidt W, Sugden B. Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. Nature. 1989;340(6232):393-397.

137. Harms PW, Vats P, Verhaegen ME, et al. The distinctive mutational spectra of polyomavirus-negative merkel cell carcinoma. Cancer Res. 2015;75(18):3720-3727.

138. Harold A, Amako Y, Hachisuka J, et al. Conversion of Sox2-dependent Merkel cell carcinoma to a differentiated neuron-like phenotype by T antigen inhibition. Proc Natl Acad Sci U S A. 2019;116(40):20104-20114.

139. Hasche D, Vinzon SE, Rosl F. Cutaneous papillomaviruses and non-melanoma skin cancer: causal agents or innocent bystanders? Front Microbiol. 2018;9:874.

140. Hasegawa H, Sawa H, Lewis MJ, et al. Thymus-derived leukemia-lymphoma in mice transgenic for the Tax gene of human T-lymphotropic virus type I. Nat Med. 2006;12(4):466-472.

141. Hayman MJ, Enrietto PJ. Cell transformation by the epidermal growth factor receptor and v-erbB. Cancer Cells. 1991;3(8):302-307.

142. Hayward WS, Neel BG, Astrin SM. Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature. 1981;290(5806):475-480.

143. Hesbacher S, Pfitzer L, Wiedorfer K, et al. RB1 is the crucial target of the Merkel cell polyomavirus Large T antigen in Merkel cell carcinoma cells. Oncotarget. 2016;7(22): 32956-32968.

144. Heslop HE, Rooney CM. Adoptive cellular immunotherapy for EBV lymphoproliferative disease. Immunol Rev. 1997;157:217-222.

145. Hocum JD, Linde I, Rae DT, Collins CP, Matern LK, Trobridge GD. Retargeted foamy virus vectors integrate less frequently near proto-oncogenes. Sci Rep. 2016;6:36610.

146. Houben R, Shuda M, Weinkam R, et al. Merkel cell polyomavirus-infected Merkel cell carcinoma cells require expression of viral T antigens. J Virol. 2010;84(14):7064-7072.

147. Hu J, Garber AC, Renne R. The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus supports latent DNA

replication in dividing cells. J Virol. 2002;76(22):11677-11687.

148. Huang E, Nocka K, Beier DR, et al. The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. Cell. 1990;63(1):225-233.

149. Hunter T, Sefton BM. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc Natl Acad Sci U S A. 1980;77(3):1311-1315.

150. Hurlin PJ, Kaur P, Smith PP, Perez-Reyes N, Blanton RA, McDougall JK. Progression of human papillomavirus type 18-immortalized human keratinocytes to a malignant phenotype. Proc Natl Acad Sci U S A. 1991;88(2):570-574.

151. Ioannou GN, Green PK, Berry K. HCV eradication induced by direct-acting antiviral agents reduces the risk of hepatocellular carcinoma. J Hepatol. 2018;68(1):25-32.

152. Izumi KM, Cahir McFarland ED, Ting AT, Riley EA, Seed B, Kieff ED. The Epstein-Barr virus oncoprotein latent membrane protein 1 engages the tumor necrosis factor receptor-associated proteins TRADD and receptor-interacting protein (RIP) but does not induce apoptosis or require RIP for NF-kappaB activation. Mol Cell Biol. 1999;19(8):5759-5767.

153. Izumi KM, Kaye KM, Kieff ED. The Epstein-Barr virus LMP1 amino acid sequence that engages tumor necrosis factor receptor associated factors is critical for primary B lymphocyte growth transformation. Proc Natl Acad Sci U S A. 1997;94(4):1447-1452.

154. Izumi KM, Kieff ED. The Epstein-Barr virus oncogene product latent membrane protein 1 engages the tumor necrosis factor receptor-associated death domain protein to mediate B lymphocyte growth transformation and activate NF-kappaB. Proc Natl Acad Sci U S A. 1997;94(23):12592-12597.

155. Jabbar SF, Abrams L, Glick A, Lambert PF. Persistence of high-grade cervical dysplasia and cervical cancer requires the continuous expression of the human papillomavirus type 16 E7 oncogene. Cancer Res. 2009;69(10):4407-4414.

156. Jansen HW, Lurz R, Bister K, Bonner TI, Mark GE, Rapp UR. Homologous cell-derived oncogenes in avian carcinoma virus MH2 and murine sarcoma virus 3611. Nature. 1984;307(5948):281-284.

157. Joazeiro CA, Wing SS, Huang H, Leverson JD, Hunter T, Liu YC. The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science. 1999;286(5438):309-312.

158. Kanwal F, Kramer J, Asch SM, Chayanupatkul M, Cao Y, El-Serag HB. Risk of hepatocellular cancer in HCV patients treated with direct-acting antiviral agents. Gastroenterology. 2017;153(4):996-1005 e1.

159. Karabadzhak AG, Petti LM, Barrera FN, et al. Two transmembrane dimers of the bovine papillomavirus E5 oncoprotein clamp the PDGF beta receptor in an active dimeric conformation. Proc Natl Acad Sci U S A. 2017;114(35):E7262-E7271.

160. Kaushansky K, Lok S, Holly RD, et al. Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. Nature. 1994;369(6481):568-571.

161. Kaye KM, Izumi KM, Kieff E. Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. Proc Natl Acad Sci U S A. 1993;90(19):9150-9154.

162. Kaye KM, Izumi KM, Mosialos G, Kieff E. The Epstein-Barr virus LMP1 cytoplasmic carboxy terminus is essential for B-lymphocyte transformation; fibroblast cocultivation complements a critical function within the terminal 155 residues. J Virol. 1995;69(2):675-683.

163. Kelly G, Bell A, Rickinson A. Epstein-Barr virus-associated Burkitt lymphomagenesis selects for downregulation of the nuclear antigen EBNA2. Nat Med. 2002;8(10):1098-1104.

164. Kelly GL, Long HM, Stylianou J, et al. An Epstein-Barr virus anti-apoptotic protein constitutively expressed in transformed cells and implicated in burkitt lymphomagenesis: the Wp/BHRF1 link. PLoS Pathog. 2009;5(3):e1000341.

165. Kennedy G, Komano J, Sugden B. Epstein-Barr virus provides a survival factor to Burkitt's lymphomas. Proc Natl Acad Sci U S A. 2003;100(24):14269-14274.

166. Kent LN, Leone G. The broken cycle: E2F dysfunction in cancer. Nat Rev Cancer. 2019;19(6):326-338.

167. Kenter GG, Welters MJ, Valentijn AR, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. N Engl J Med. 2009;361(19):1838-1847.

168. Kirnbauer R, Booy F, Cheng N, Lowy DR, Schiller JT. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proc Natl Acad Sci U S A. 1992;89(24):12180-12184.

169. Klimm B, Franklin J, Stein H, et al. Lymphocyte-depleted classical Hodgkin's lymphoma: a comprehensive analysis from the German Hodgkin study group. J Clin Oncol. 2011;29(29):3914-3920.

170. Koch CA, Anderson D, Moran MF, Ellis C, Pawson T. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science. 1991;252(5006):668-674.

171. Kolch W, Heidecker G, Lloyd P, Rapp UR. Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. Nature. 1991;349(6308):426-428.

172. Koutsky LA, Ault KA, Wheeler CM, et al. A controlled trial of a human papillomavirus type 16 vaccine. N Engl J Med. 2002;347(21):1645-1651.

173. Krause D. Therapy for Wiskott-Aldrich syndrome: benefits and risks. Hematologist. 2011;8:10.

174. Krishnan HH, Naranatt PP, Smith MS, Zeng L, Bloomer C, Chandran B. Concurrent expression of latent and a limited number of lytic genes with immune modulation and antiapoptotic function by Kaposi's sarcoma-associated herpesvirus early during infection of primary endothelial and fibroblast cells and subsequent decline of lytic gene expression. J Virol. 2004;78(7):3601-3620.

175. Kulwichit W, Edwards RH, Davenport EM, Baskar JF, Godfrey V, Raab-Traub N. Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice. Proc Natl Acad Sci U S A. 1998;95(20):11963-11968.

176. Lan K, Kuppers DA, Robertson ES. Kaposi's sarcoma-associated herpesvirus reactivation is regulated by interaction of latency-associated nuclear antigen with recombination signal sequence-binding protein Jkappa, the major downstream effector of the Notch signaling pathway. J Virol. 2005;79(6):3468-3478.

177. Lane DP, Crawford LV. T antigen is bound to a host protein in SV40-transformed cells. Nature. 1979;278(5701):261-263.

178. Largaespada DA, Shaughnessy JD Jr, Jenkins NA, Copeland NG. Retroviral integration at the Evi-2 locus in BXH-2 myeloid leukemia cell lines disrupts Nf1 expression without changes in steady-state Ras-GTP levels. J Virol. 1995;69(8):5095-5102.

179. Lau L, Gray EE, Brunette RL, Stetson DB. DNA tumor virus oncogenes antagonize the cGAS-STING DNA-sensing pathway. Science. 2015;350(6260):568-571.

180. Lee H, Guo J, Li M, et al. Identification of an immunoreceptor tyrosine-based activation motif of K1 transforming protein of Kaposi's sarcoma-associated herpesvirus. Mol Cell Biol. 1998;18(9):5219-5228.

181. Lei J, Ploner A, Elfstrom KM, et al. HPV Vaccination and the risk of invasive cervical cancer. N Engl J Med. 2020;383(14):1340-1348.

182. Lerat H, Honda M, Beard MR, et al. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology. 2002;122(2):352-365.

183. Li J, Wang X, Diaz J, Tsang SH, Buck CB, You J. Merkel cell polyomavirus large T antigen disrupts host genomic integrity and inhibits cellular proliferation. J Virol. 2013;87(16):9173-9188.

184. Li JP, D'Andrea AD, Lodish HF, Baltimore D. Activation of cell growth by binding of Friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. Nature. 1990;343(6260):762-764.

185. Li X, Fasano R, Wang E, Yao KT, Marincola FM. HLA associations with nasopharyngeal carcinoma. Curr Mol Med. 2009;9(6):751-765.

186. Lim C, Sohn H, Gwack Y, Choe J. Latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) binds ATF4/CREB2 and inhibits its transcriptional activation activity. J Gen Virol. 2000;81(Pt 11):2645-2652.

187. Ling PD, Rawlins DR, Hayward SD. The Epstein-Barr virus immortalizing protein EBNA-2 is targeted to DNA by a cellular enhancer-binding protein. Proc Natl Acad Sci U S A. 1993;90(20):9237-9241.

188. Linzer DI, Levine AJ. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell. 1979;17(1):43-52.

189. Liu J, Martin HJ, Liao G, Hayward SD. The Kaposi's sarcoma-associated herpesvirus LANA protein stabilizes and activates c-Myc. J Virol. 2007;81(19):10451-10459.

190. Lowenstein EJ, Daly RJ, Batzer AG, et al. The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell. 1992;70(3):431-442.

191. Lu J, Murakami M, Verma SC, et al. Epstein-Barr virus nuclear antigen 1 (EBNA1) confers resistance to apoptosis in EBV-positive B-lymphoma cells through up-regulation of survivin. Virology. 2011;410(1):64-75.

192. Lubyova B, Kellum MJ, Frisancho JA, Pitha PM. Stimulation of c-Myc transcriptional activity by vIRF-3 of Kaposi sarcoma-associated herpesvirus. J Biol Chem. 2007;282(44):31944-31953.

193. Maier S, Staffler G, Hartmann A, et al. Cellular target genes of Epstein-Barr virus nuclear antigen 2. J Virol. 2006;80(19):9761-9771.

194. Mainou BA, Everly DN Jr, Raab-Traub N. Epstein-Barr virus latent membrane protein 1 CTAR1 mediates rodent and human fibroblast transformation through activation of PI3K. Oncogene. 2005;24(46):6917-6924.

195. Martin GS. Rous sarcoma virus: a function required for the maintenance of the transformed state. Nature. 1970;227(5262):1021-1023.

196. Matsuoka M, Green PL. The HBZ gene, a key player in HTLV-1 pathogenesis. Retrovirology. 2009;6:71.

197. Mayer BJ, Hanafusa H. Association of the v-crk oncogene product with phosphotyrosine-containing proteins and protein kinase activity. Proc Natl Acad Sci U S A. 1990;87(7):2638-2642.

198. Montaner S. Akt/TSC/mTOR activation by the KSHV G protein-coupled receptor: emerging insights into the molecular oncogenesis and treatment of Kaposi's sarcoma. Cell Cycle. 2007;6(4):438-443.

199. Moran MF, Koch CA, Anderson D, et al. Src homology region 2 domains direct protein-protein interactions in signal transduction. Proc Natl Acad Sci U S A. 1990;87(21):8622-8626.

200. Moriya K, Fujie H, Shintani Y, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med. 1998;4(9):1065-1067.

201. Mosialos G, Birkenbach M, Yalamanchili R, VanArsdale T, Ware C, Kieff E. The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. Cell. 1995;80(3):389-399.

202. Muromoto R, Okabe K, Fujimuro M, et al. Physical and functional interactions between STAT3 and Kaposi's sarcoma-associated herpesvirus-encoded LANA. FEBS Lett. 2006;580(1):93-98.

203. Murphy PM. Pirated genes in Kaposi's sarcoma. Nature. 1997;385(6614):296-297, 299.

204. Nador RG, Cesarman E, Chadburn A, et al. Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. Blood. 1996;88(2):645-656.

205. Nakabeppu Y, Ryder K, Nathans D. DNA binding activities of three murine Jun proteins: stimulation by Fos. Cell. 1988;55(5):907-915.

206. Nathans D. Restriction endonucleases, simian virus 40, and the new genetics. Science. 1979;206(4421):903-909.

207. Negri E, Little D, Boiocchi M, La Vecchia C, Franceschi S. B-cell non-Hodgkin's lymphoma and hepatitis C virus infection: a systematic review. Int J Cancer. 2004;111(1):1-8.

208. Nienhuis AW, Dunbar CE, Sorrentino BP. Genotoxicity of retroviral integration in hematopoietic cells. Mol Ther. 2006;13(6):1031-1049.

209. Niller HH, Wolf H, Minarovits J. Viral hit and run-oncogenesis: genetic and epigenetic scenarios. Cancer Lett. 2011;305(2):200-217.

210. Niman HL. Antisera to a synthetic peptide of the sis viral oncogene product recognize human platelet-derived growth factor. Nature. 1984;307(5947):180-183.

211. Oksenhendler E, Boulanger E, Galicier L, et al. High incidence of Kaposi sarcoma-associated herpesvirus-related non-Hodgkin lymphoma in patients with HIV infection and multicentric Castleman disease. Blood. 2002;99(7):2331-2336.

212. Ottinger M, Christalla T, Nathan K, Brinkmann MM, Viejo-Borbolla A, Schulz TF. Kaposi's sarcoma-associated herpesvirus LANA-1 interacts with the short variant of BRD4 and releases cells from a BRD4- and BRD2/RING3-induced G1 cell cycle arrest. J Virol. 2006;80(21):10772-10786.

213. Pal A, Kundu R. Human papillomavirus E6 and E7: the cervical cancer hallmarks and targets for therapy. Front Microbiol. 2019;10:3116.

214. Park DE, Cheng J, McGrath JP, et al. Merkel cell polyomavirus activates LSD1-mediated blockade of non-canonical BAF to regulate transformation and tumorigenesis. Nat Cell Biol. 2020;22(5):603-615.

215. Park J, Seo T, Hwang S, Lee D, Gwack Y, Choe J. The K-bZIP protein from Kaposi's sarcoma-associated herpesvirus interacts with p53 and represses its transcriptional activity. J Virol. 2000;74(24):11977-11982.

216. Parker GA, Crook T, Bain M, Sara EA, Farrell PJ, Allday MJ. Epstein-Barr virus nuclear antigen (EBNA)3C is an immortalizing oncoprotein with similar properties to adenovirus E1A and papillomavirus E7. Oncogene. 1996;13(12):2541-2549.

217. Paschos K, Smith P, Anderton E, Middeldorp JM, White RE, Allday MJ. Epstein-barr virus latency in B cells leads to epigenetic repression and CpG methylation of the tumour suppressor gene Bim. PLoS Pathog. 2009;5(6):e1000492.

218. Pathmanathan R, Prasad U, Sadler R, Flynn K, Raab-Traub N. Clonal proliferations of cells infected with Epstein-Barr virus in preinvasive lesions related to nasopharyngeal carcinoma. N Engl J Med. 1995;333(11):693-698.

219. Paulson KG, Lemos BD, Feng B, et al. Array-CGH reveals recurrent genomic changes in Merkel cell carcinoma including amplification of L-Myc. J Invest Dermatol. 2009;129(6):1547-1555.

220. Peng M, Lundgren E. Transient expression of the Epstein-Barr virus LMP1 gene in human primary B cells induces cellular activation and DNA synthesis. Oncogene. 1992;7(9):1775-1782.

221. Peter M, Rosty C, Couturier J, Radvanyi F, Teshima H, Sastre-Garau X. MYC activation associated with the integration of HPV DNA at the MYC locus in genital tumors. Oncogene. 2006;25(44):5985-5993.

222. Petti L, DiMaio D. Stable association between the bovine papillomavirus E5 transforming protein and activated platelet-derived growth factor receptor in transformed mouse cells. Proc Natl Acad Sci U S A. 1992;89(15):6736-6740.

223. Petti L, Nilson LA, DiMaio D. Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein. EMBO J. 1991;10(4):845-855.

224. Pipas JM. Common and unique features of T antigens encoded by the polyomavirus group. J Virol. 1992;66(7):3979-3985.

225. Pipas JM. DNA tumor viruses and their contributions to molecular biology. J Virol. 2019;93(9):e01524-18.

226. Plummer M, de Martel C, Vignat J, Ferlay J, Bray F, Franceschi S. Global burden of cancers attributable to infections in 2012: a synthetic analysis. Lancet Glob Health. 2016;4(9):e609-e616.

227. Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc Natl Acad Sci U S A. 1980;77(12):7415-7419.

228. Prakash O, Swamy OR, Peng X, et al. Activation of Src kinase Lyn by the Kaposi sarcoma-associated herpesvirus K1 protein: implications for lymphomagenesis. Blood. 2005;105(10):3987-3994.

229. Prakash O, Tang ZY, Peng X, et al. Tumorigenesis and aberrant signaling in transgenic mice expressing the human herpesvirus-8 K1 gene. J

Natl Cancer Inst. 2002;94(12):926-935.

230. Qu C, Chen T, Fan C, et al. Efficacy of neonatal HBV vaccination on liver cancer and other liver diseases over 30-year follow-up of the Qidong hepatitis B intervention study: a cluster randomized controlled trial. PLoS Med. 2014;11(12):e1001774.

231. Rapp UR, Goldsborough MD, Mark GE, et al. Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. Proc Natl Acad Sci U S A. 1983;80(14):4218-4222.

232. Rauscher FJ, 3rd, Cohen DR, Curran T, et al. Fos-associated protein p39 is the product of the jun proto-oncogene. Science. 1988;240(4855):1010-1016.

233. Ressing ME, Horst D, Griffin BD, et al. Epstein-Barr virus evasion of CD8(+) and CD4(+) T cell immunity via concerted actions of multiple gene products. Semin Cancer Biol. 2008;18(6):397-408.

234. Reya T, Clevers H. Wnt signalling in stem cells and cancer. Nature. 2005;434(7035):843-850.

235. Ringehan M, McKeating JA, Protzer U. Viral hepatitis and liver cancer. Philos Trans R Soc Lond B Biol Sci. 2017;372(1732):20160274.

236. Robbins HA, Shiels MS, Pfeiffer RM, Engels EA. Epidemiologic contributions to recent cancer trends among HIV-infected people in the United States. AIDS. 2014;28(6):881-890.

237. Roueche H. [Mobile photofluorographic services of regional Social Security Offices]. Concours Med. 1952;74(45):3813-3814.

238. Rous P. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. J Exp Med. 1911;13(4):397-411.

239. Rovedo M, Longnecker R. Epstein-Barr virus latent membrane protein 2A preferentially signals through the Src family kinase Lyn. J Virol. 2008;82(17):8520-8528.

240. Rozakis-Adcock M, McGlade J, Mbamalu G, et al. Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. Nature. 1992;360(6405):689-692.

241. Sablina AA, Hahn WC. SV40 small T antigen and PP2A phosphatase in cell transformation. Cancer Metastasis Rev. 2008;27(2):137-146.

242. Saito I, Miyamura T, Ohbayashi A, et al. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci U S A. 1990;87(17):6547-6549.

243. Sap J, Munoz A, Damm K, et al. The c-erb-A protein is a high-affinity receptor for thyroid hormone. Nature. 1986;324(6098):635-640.

244. Satou Y, Yasunaga J, Zhao T, et al. HTLV-1 bZIP factor induces T-cell lymphoma and systemic inflammation in vivo. PLoS Pathog. 2011;7(2):e1001274.

245. Schaffhausen BS, Roberts TM. Lessons from polyoma middle T antigen on signaling and transformation: a DNA tumor virus contribution to the war on cancer. Virology. 2009;384(2):304-316.

246. Schiffman M, Doorbar J, Wentzensen N, et al. Carcinogenic human papillomavirus infection. Nat Rev Dis Primers. 2016;2:16086.

247. Schiller JT, Lowy DR. Vaccines to prevent infections by oncoviruses. Annu Rev Microbiol. 2010;64:23-41.

248. Schlegel R, Wade-Glass M, Rabson MS, Yang YC. The E5 transforming gene of bovine papillomavirus encodes a small, hydrophobic polypeptide. Science. 1986;233(4762):464-467.

249. Scholle F, Bendt KM, Raab-Traub N. Epstein-Barr virus LMP2A transforms epithelial cells, inhibits cell differentiation, and activates Akt. J Virol. 2000;74(22):10681-10689.

250. Scolnick EM, Howk RS, Anisowicz A, Peebles PT, Scher CD, Parks WP. Separation of sarcoma virus-specific and leukemia virus-specific genetic sequences of Moloney sarcoma virus. Proc Natl Acad Sci U S A. 1975;72(11):4650-4654.

251. Selten G, Cuypers HT, Zijlstra M, Melief C, Berns A. Involvement of c-myc in MuLV-induced T cell lymphomas in mice: frequency and mechanisms of activation. EMBO J. 1984;3(13):3215-3222.

252. Shah KM, Young LS. Epstein-Barr virus and carcinogenesis: beyond Burkitt's lymphoma. Clin Microbiol Infect. 2009;15(11):982-988.

253. Sheiness D, Bister K, Moscovici C, Fanshier L, Gonda T, Bishop JM. Avian retroviruses that cause carcinoma and leukemia: identification of nucleotide sequences associated with pathogenicity. J Virol. 1980;33(3):962-968.

254. Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER. The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell. 1985;41(3):665-676.

255. Shiels MS, Islam JY, Rosenberg PS, Hall HI, Jacobson E, Engels EA. Projected cancer incidence rates and burden of incident cancer cases in HIV-infected adults in the United States Through 2030. Ann Intern Med. 2018;168(12):866-873.

256. Shuda M, Feng H, Kwun HJ, et al. T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus. Proc Natl Acad Sci U S A. 2008;105(42):16272-16277.

257. Shuda M, Guastafierro A, Geng X, et al. Merkel cell polyomavirus small T antigen induces cancer and embryonic merkel cell proliferation in a transgenic mouse model. PLoS One. 2015;10(11):e0142329.

258. Sigel K, Park L, Justice A. HIV and cancer in the Veterans Health Administration System. Semin Oncol. 2019;46(4-5):334-340.

259. Sin SH, Roy D, Wang L, et al. Rapamycin is efficacious against primary effusion lymphoma (PEL) cell lines in vivo by inhibiting autocrine signaling. Blood. 2007;109(5):2165-2173.

260. Singh M, Kumar V. Transgenic mouse models of hepatitis B virus-associated hepatocellular carcinoma. Rev Med Virol. 2003;13(4):243-253.

261. Skalska L, White RE, Franz M, Ruhmann M, Allday MJ. Epigenetic repression of p16(INK4A) by latent Epstein-Barr virus requires the interaction of EBNA3A and EBNA3C with CtBP. PLoS Pathog. 2010;6(6):e1000951.

262. Smirnova IS, Aksenov ND, Kashuba EV, et al. Hepatitis C virus core protein transforms murine fibroblasts by promoting genomic instability. Cell Oncol. 2006;28(4):177-190.

263. Smith LM, Strumpf EC, Kaufman JS, Lofters A, Schwandt M, Levesque LE. The early benefits of human papillomavirus vaccination on cervical dysplasia and anogenital warts. Pediatrics. 2015;135(5):e1131-e1140.

264. Sodhi A, Montaner S, Patel V, et al. Akt plays a central role in sarcomagenesis induced by Kaposi's sarcoma herpesvirus-encoded G protein-coupled receptor. Proc Natl Acad Sci U S A. 2004;101(14):4821-4826.

265. Soulier J, Grollet L, Oksenhendler E, et al. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. Blood. 1995;86(4):1276-1280.

266. Spender LC, Cornish GH, Sullivan A, Farrell PJ. Expression of transcription factor AML-2 (RUNX3, CBF(alpha)-3) is induced by Epstein-Barr virus EBNA-2 and correlates with the B-cell activation phenotype. J Virol. 2002;76(10):4919-4927.

267. Spurgeon ME, Cheng J, Bronson RT, Lambert PF, DeCaprio JA. Tumorigenic activity of merkel cell polyomavirus T antigens expressed in the stratified epithelium of mice. Cancer Res. 2015;75(6):1068-1079.

268. Starrett GJ, Buck CB. The case for BK polyomavirus as a cause of bladder cancer. Curr Opin Virol. 2019;39:8-15.

269. Starrett GJ, Marcelus C, Cantalupo PG, et al. Merkel cell polyomavirus exhibits dominant control of the tumor genome and transcriptome in virus-associated merkel cell carcinoma. MBio. 2017;8(1):e02079-16.

270. Starrett GJ, Thakuria M, Chen T, et al. Clinical and molecular characterization of virus-positive and virus-negative Merkel cell carcinoma. Genome Med. 2020;12(1):30.

271. Stehelin D, Varmus HE, Bishop JM, Vogt PK. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. Nature. 1976;260(5547):170-173.

272. Stein S, Ott MG, Schultze-Strasser S, et al. Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. Nat Med. 2010;16(2):198-204.

273. Stoye JP, Moroni C, Coffin JM. Virological events leading to spontaneous AKR thymomas. J Virol. 1991;65(3):1273-1285.

274. Straight SW, Hinkle PM, Jewers RJ, McCance DJ. The E5 oncoprotein of human papillomavirus type 16 transforms fibroblasts and effects the downregulation of the epidermal growth factor receptor in keratinocytes. J Virol. 1993;67(8):4521-4532.

275. Summers J, Mason WS. Replication of the genome of a hepatitis B—like virus by reverse transcription of an RNA intermediate. Cell. 1982;29(2):403-415.

276. Sun Q, Zachariah S, Chaudhary PM. The human herpes virus 8-encoded viral FLICE-inhibitory protein induces cellular transformation via NF-kappaB activation. J Biol Chem. 2003;278(52):52437-52445.

277. Sung WK, Zheng H, Li S, et al. Genome-wide survey of recurrent HBV integration in hepatocellular carcinoma. Nat Genet. 2012;44(7):765-769.

278. Swanstrom R, Parker RC, Varmus HE, Bishop JM. Transduction of a cellular oncogene: the genesis of Rous sarcoma virus. Proc Natl Acad Sci U S A. 1983;80(9):2519-2523.

279. Swanton C, Mann DJ, Fleckenstein B, Neipel F, Peters G, Jones N. Herpes viral cyclin/Cdk6 complexes evade inhibition by CDK inhibitor proteins. Nature. 1997;390(6656):184-187.

280. Sylla BS, Temin HM. Activation of oncogenicity of the c-rel proto-oncogene. Mol Cell Biol. 1986;6(12):4709-4716.

281. Takeya T, Hanafusa H. Structure and sequence of the cellular gene homologous to the RSV src gene and the mechanism for generating the transforming virus. Cell. 1983;32(3):881-890.

282. Thorley-Lawson DA, Allday MJ. The curious case of the tumour virus: 50 years of Burkitt's lymphoma. Nat Rev Microbiol. 2008;6(12):913-924.

283. Tomlinson CC, Damania B. The K1 protein of Kaposi's sarcoma-associated herpesvirus activates the Akt signaling pathway. J Virol. 2004;78(4):1918-1927.

284. Totoki Y, Tatsuno K, Covington KR, et al. Trans-ancestry mutational landscape of hepatocellular carcinoma genomes. Nat Genet. 2014;46(12):1267-1273.

285. Trahey M, McCormick F. A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. Science. 1987;238(4826):542-545.

286. Treisman R, Novak U, Favaloro J, Kamen R. Transformation of rat cells by an altered polyoma virus genome expressing only the middle-T protein. Nature. 1981;292(5824): 595-600.

287. Uchida J, Yasui T, Takaoka-Shichijo Y, et al. Mimicry of CD40 signals by Epstein-Barr virus LMP1 in B lymphocyte responses. Science. 1999;286(5438):300-303.

288. Valentine R, Dawson CW, Hu C, et al. Epstein-Barr virus-encoded EBNA1 inhibits the canonical NF-kappaB pathway in carcinoma cells by inhibiting IKK phosphorylation. Mol Cancer. 2010;9:1.

289. Vennstrom B, Sheiness D, Zabielski J, Bishop JM. Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29. J Virol. 1982;42(3):773-779.

290. Verhaegen ME, Mangelberger D, Harms PW, et al. Merkel cell polyomavirus small T antigen initiates merkel cell carcinoma-like tumor development in mice. Cancer Res. 2017;77(12):3151-3157.

291. Verma SC, Borah S, Robertson ES. Latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus up-regulates transcription of human telomerase reverse transcriptase promoter through interaction with transcription factor Sp1. J Virol. 2004;78(19):10348-10359.

292. Viarisio D, Muller-Decker K, Accardi R, et al. Beta HPV38 oncoproteins act with a hit-and-run mechanism in ultraviolet radiation-induced skin carcinogenesis in mice. PLoS Pathog. 2018;14(1):e1006783.

293. Wang D, Liebowitz D, Kieff E. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell. 1985;43(3 Pt 2):831-840.

294. Wang F, Gregory CD, Rowe M, et al. Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23. Proc Natl Acad Sci U S A. 1987;84(10):3452-3456.

295. Wang HW, Sharp TV, Koumi A, Koentges G, Boshoff C. Characterization of an anti-apoptotic glycoprotein encoded by Kaposi's sarcoma-associated herpesvirus which resembles a spliced variant of human survivin. EMBO J. 2002;21(11):2602-2615.

296. Warburton A, Markowitz TE, Katz JP, Pipas JM, McBride AA. Recurrent integration of human papillomavirus genomes at transcriptional regulatory hubs. *NPJ Genom Med*. 2021;101:doi 10.1038/s41525-021-00264-4-y

297. Wei F, Gan J, Wang C, Zhu C, Cai Q. Cell cycle regulatory functions of the KSHV oncoprotein LANA. Front Microbiol. 2016;7:334.

298. Weinberger C, Thompson CC, Ong ES, Lebo R, Gruol DJ, Evans RM. The c-erb-A gene encodes a thyroid hormone receptor. Nature. 1986;324(6098):641-646.

299. Weitzman MD, Carson CT, Schwartz RA, Lilley CE. Interactions of viruses with the cellular DNA repair machinery. DNA Repair (Amst). 2004;3(8-9):1165-1173.

300. Wen KW, Wang L, Menke JR, Damania B. Cancers associated with human gammaherpesviruses. FEBS J. 2021; doi: 10.1111/febs.16206

301. White MK, Pagano JS, Khalili K. Viruses and human cancers: a long road of discovery of molecular paradigms. Clin Microbiol Rev. 2014;27(3):463-481.

302. Witte ON, Goff S, Rosenberg N, Baltimore D. A transformation-defective mutant of Abelson murine leukemia virus lacks protein kinase activity. Proc Natl Acad Sci U S A. 1980;77(8):4993-4997.

303. Wood VH, O'Neil JD, Wei W, Stewart SE, Dawson CW, Young LS. Epstein-Barr virus-encoded EBNA1 regulates cellular gene transcription and modulates the STAT1 and TGFbeta signaling pathways. Oncogene. 2007;26(28):4135-4147.

304. Wu CY, Lin JT, Ho HJ, et al. Association of nucleos(t)ide analogue therapy with reduced risk of hepatocellular carcinoma in patients with chronic hepatitis B: a nationwide cohort study. Gastroenterology. 2014;147(1):143-151 e5.

305. Xu W, Harrison SC, Eck MJ. Three-dimensional structure of the tyrosine kinase c-Src. Nature. 1997;385(6617):595-602.

306. Yi F, Saha A, Murakami M, et al. Epstein-Barr virus nuclear antigen 3C targets p53 and modulates its transcriptional and apoptotic activities. Virology. 2009;388(2):236-247.

307. Yoshida M, Miyoshi I, Hinuma Y. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. Proc Natl Acad Sci U S A. 1982;79(6):2031-2035.

308. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer. 2004;4(10):757-768.

309. Yu MC, Mo CC, Chong WX, Yeh FS, Henderson BE. Preserved foods and nasopharyngeal carcinoma: a case-control study in Guangxi, China. Cancer Res. 1988;48(7):1954-1959.

310. Zanetti AR, Van Damme P, Shouval D. The global impact of vaccination against hepatitis B: a historical overview. Vaccine. 2008;26(49):6266-6273.

311. Zapatka M, Borozan I, Brewer DS, et al. The landscape of viral associations in human cancers. Nat Genet. 2020;52(3):320-330.

312. Zhang SM, Cai WL, Liu X, et al. KDM5B promotes immune evasion by recruiting SETDB1 to silence retroelements. Nature. 2021;598(7882):682-687.

313. Zhang XF, Settleman J, Kyriakis JM, et al. Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1.

Nature. 1993;364(6435):308-313.

314. Zhao LH, Liu X, Yan HX, et al. Genomic and oncogenic preference of HBV integration in hepatocellular carcinoma. Nat Commun. 2016;7:12992.

315. Zhou J, Sun XY, Stenzel DJ, Frazer IH. Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. Virology. 1991;185(1):251-257.

316. Zsebo KM, Williams DA, Geissler EN, et al. Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. Cell. 1990;63(1):213-224.

317. zur Hausen H. Papillomavirus infections—a major cause of human cancers. Biochim Biophys Acta. 1996;1288(2):F55-F78.

CHAPTER 12

Evolution of Viral Proteins

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Virus hallmark proteins

RNA-dependent RNA polymerase and reverse transcriptase

Family B DNA polymerases

Rolling circle replication initiation endonuclease

Palm/RRM domain, the core of the virus replication machinery

Helicases and genome packaging NTPases

Virus capsid proteins

Evolutionary roots of the virus hallmark proteins and implications for the origin of viruses

Moderately conserved proteins involved in core virus functions

P-loop enzymes

Proteases

DNA-dependent RNA polymerases and transcription factors

Capping enzymes

Lineage-specific virus proteins involved in core processes

Virus proteins involved in virus-host interactions

Dark matter of virus proteomes

Exaptation and recruitment of host proteins as major routes of virus protein evolution

Concluding remarks: organization of the virus pan-proteome and challenges in its analysis

Virus genomes span about three orders of magnitude in genome size, from <2 kilobases (kb) in the smallest RNA and single-stranded (ss) DNA viruses to more than 2 megabases (Mb) in the giant pandoraviruses. However, from the dwarfs to the giants of the virus world, viruses share a signature feature, the high density of protein-coding genes. Although some viruses encode multiple RNA species, such as tRNAs as well as other noncoding RNAs, at least 90% of the genome sequence of any virus is dedicated to protein coding.[120] Viral proteins are responsible for essential roles in virus reproduction, such as the formation of virions and at least some functions of the virus genome replication. Typically, the proteins that are essential for virus reproduction are shared by large groups of viruses (hereafter, virus hallmark proteins, or VHP).[96,132] The simplest viruses with smallest genomes encode primarily or exclusively VHPs.[136] Viruses with larger genomes encode a great variety of additional proteins that are primarily involved in virus-host interactions, in particular, counteracting host defenses. The genomes of giant viruses encode hundreds of such ancillary proteins that remain poorly characterized.[16,126,154]

The key feature of virus genome evolution is its much higher pace compared to the genomes of cellular life forms. The evolution rates differ widely among viruses, but in many cases,[51,203,221] even a single family of viruses can explore a larger region of the sequence space in its evolution than an entire domain of cellular life forms.[51,203,221] This fast evolution of virus proteins presents major challenges for the analysis of the evolutionary relationships among virus proteins and consequently, among different groups of viruses.[36] Even VHPs often can be identified only by applying the most sensitive sequence comparison approaches and/or methods for protein structure comparison. As for ancillary proteins, virus proteomes remain replete with "dark matter," proteins for which neither structure nor function can be predicted without direct experiment.

Evolution of viruses involves extensive gene exchange with the hosts.[98,123] Therefore, the evolutionary histories of virus proteins are diverse and often intricate. For the VHPs, a long history of evolution within the virosphere is traceable, and origin from specific cellular homologs at the start of that history is apparent for some but not other of these key viral proteins. For the ancillary virus proteins, diverse evolutionary scenarios can be reconstructed. Apart from the dark matter, these proteins span a wide range of sequence and structure similarity to cellular homologs, suggestive of both different modes of evolution and different timing of gene capture by viruses. On many occasions, adaptation of a captured host protein by a virus represents exaptation,[80,81] a distinct mode of evolution whereby a protein acquired by a virus changes its function (eg, an enzyme is inactivated and repurposed for a structural role), resulting in major changes, at least at the amino acid sequence level. In other cases, the functional and structural modification is less dramatic, for example, when a component of a cellular molecular machinery is exploited by a virus as a dominant negative inhibitor of the respective host function, such as programmed cell death.

In this chapter, we present an overview of the currently available data and concepts on the origins and evolution of different classes of virus proteins. Our description of the evolutionary trends among virus proteins is structured in accordance with the recently developed

comprehensive hierarchical taxonomy of viruses.[95,127] This taxonomy organizes the virosphere throughout the multiple taxonomic levels, down from the top rank of realms (Table 12.1; Fig. 12.1).

TABLE 12.1 The top levels of virus taxonomy

Realm	Families	Hosts	Genome Type	Defining Properties
Adnaviria	4	Archaea	dsDNA	Filamentous virions with linear A-form dsDNA genomes
Duplodnaviria	50	Archaea, bacteria, eukaryotes	dsDNA	Viruses with icosahedral capsids made of HK97-fold MCPs
Monodnaviria	20	Archaea, bacteria, eukaryotes	ssDNA and some dsDNA	Viruses with ssDNA genomes replicated using rolling-circle mechanism and encoding HUH superfamily encoducleases
Riboviria	117	Bacteria and eukaryotes	RNA, RT-RNA, RT-DNA	RNA and reverse-transcribing viruses encoding palm-domain RdRPs or RTs
Ribozyviria	1	Eukaryotes	(–)RNA	Viruses with circular (–)RNA genomes with ribozyme features and lacking RdRP gene
Varidnaviria	18	Archaea, bacteria, eukaryotes	dsDNA and some ssDNA	Viruses with icosahedral virions made of DJR-MCPs and derived viruses

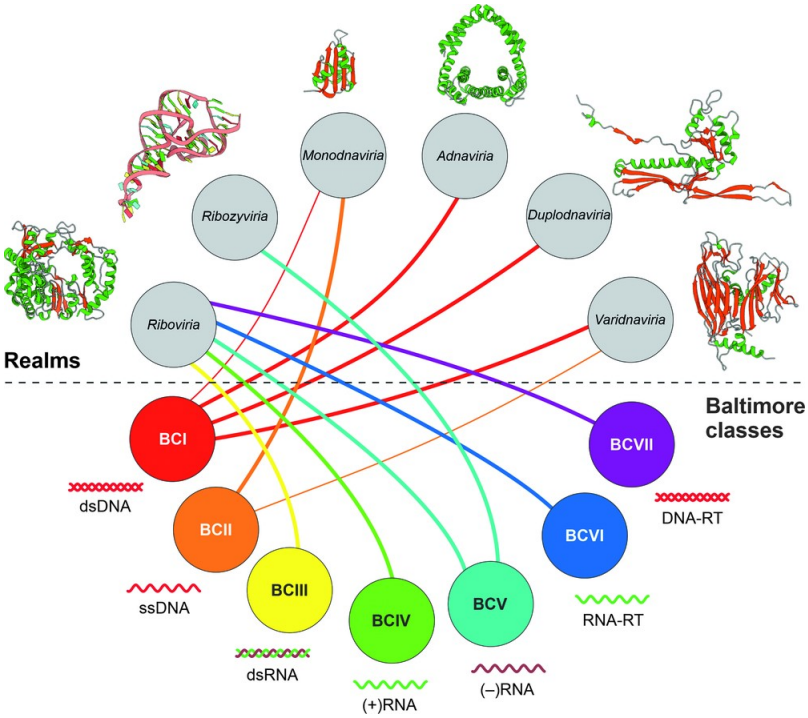

FIGURE 12.1 **The Baltimore classes and monophyletic realms of viruses**. The connections between the Baltimore classes and the virus realms are shown by colored edges. *Thick lines* denote major associations, and *thin lines* denote exceptional cases. For each virus realm, a ribbon diagram of a hallmark structure is shown as follows: *Riboviria*, poliovirus RdRP (PDB id: 1ra7); *Ribozyviria*, HDV ribozyme (PDB id: 4prf); *Monodnaviria*, porcine circovirus 2 rolling circle replication initiation endonuclease domain (PDB id: 5xor); *Adnaviria*, major capsid protein of Sulfolobus islandicus rod-shaped virus 2 (PDB id: 3j9x); *Duplodnaviria*, major capsid protein of bacteriophage HK97 (PDB id: 1ohg); *Varidnaviria*, double jelly roll major capsid protein of bacteriophage PRD1 (PDB id: 1hx6). The protein structures are colored by secondary structure: α-helices, *green*; β-strands, *red*. (Used with permission of American Society for Microbiology from Koonin EV, Krupovic M, Agol VI. The Baltimore Classification of viruses 50 years later: how does it stand in the light of virus evolution? *Microbiol Mol Biol Rev*. 2021;85(3):e0005321; permission conveyed through Copyright Clearance Center, Inc.)

Virus Hallmark Proteins

Proteins that are shared by a broad range of viruses are hallmarks of the virus state.[132] Indeed, these proteins are responsible for the key functions in virus reproduction, namely, virion formation and replication of virus genomes. These two categories of functions differ in that the major components of virions are encoded in any virus genomes, whereas genome replication, at least, in viruses with DNA genomes, is mediated by interacting virus and host proteins, in widely different proportions across various groups of viruses.[68,128,136,212,256]

The VHPs are difficult to define formally, and some degree of arbitrariness in this definition is hard to avoid. Nevertheless, the number of VHPs, obviously, is small, no more than 20, depending on the criteria applied (Table 12.2). Several of these proteins are “super-VHPs,” which connect different Baltimore classes of viruses, that is, viruses that encapsidate different forms of nucleic acids (single-stranded or double-stranded RNA or DNA) and accordingly employ distinct routes of genome expression (Fig. 12.1).[19,129] Other VHPs bring together multiple, diverse groups of viruses within a single Baltimore class. Furthermore, deeper connections among the VHPs exist at the level of domain folds. In the description that follows, we group the VHPs by such deep commonalities that imply ancient common origin. In the currently adopted high level taxonomy of

viruses, the VHPs serve as signatures defining six virus realms (Fig. 12.1), the highest level of the taxonomic hierarchy, and to a large extent, the kingdoms, the next level taxa.[127,128,143]

TABLE 12.2 Virus hallmark proteins

Protein	Function	Distribution
RNA-dependent RNA polymerase	Replicase of RNA viruses	*Orthornavirae*
Reverse transcriptase	Replicase of all RT viruses	*Pararnavirae*
Rolling circle replication endonuclease	Initiator of rolling circle replication	*Monodnaviria*, some *Duplodnaviria*, *Varidnaviria*, and *Adnaviria*
Family B DNA polymerase	Replication of dsDNA genomes	Many *Duplodnaviria*, *Varidnaviria*, *Naldaviricetes*, several families of archaeal viruses
Superfamily 3 helicase	Unwinding or RNA and DNA genomes during replication	Many *Riboviria*, *Duplodnaviria*, *Varidnaviria*, *Monodnaviria*
Archaeo-eukaryotic primase	Priming of DNA synthesis	Many *Duplodnaviria*, *Varidnaviria*, *Naldaviricetes*
Single jelly roll capsid protein	Major and minor capsid proteins	Many *Monodnaviria*, *Orthornavirae*, *Helvetiavirae*
Double jelly roll capsid protein	Major capsid protein	Majority of *Bamfordvirae*
HK97-like capsid protein	Major capsid protein	*Duplodnaviria*
FtsK-like ATPase	Genome packaging ATPase	Most *Varidnaviria*, *Loebvirae* (filamentous phages)
Terminase, large subunit	Genome packaging ATPase and nuclease	*Duplodnaviria*
Portal protein	Structural protein essential for genome packaging	*Duplodnaviria*

RNA-Dependent RNA Polymerase and Reverse Transcriptase

With few exceptions, RNA replication does not occur in cells, and accordingly, the replication of viruses with RNA genomes that constitute the virus realm *Riboviria* is catalyzed by virus-encoded enzymes (Fig. 12.1). Within the kingdom *Orthornavirae*, which includes viruses with positive-sense (+) RNA, negative-sense (−) RNA, and dsRNA genomes that have no DNA stage in their replication cycle, the enzyme responsible for the genome replication (and transcription, in viruses that produce subgenomic mRNAs) is the RNA-dependent RNA polymerase (RdRP).[196,239,240] The RdRP is the signature of *Orthornavirae*, the only VHP that is universally conserved in the viruses of this kingdom. The second kingdom under *Riboviria* is *Pararnavirae*, which consists of viruses with (+)RNA or dsDNA genomes that include a reverse transcription step in their replication cycles. The signature VHP of *Pararnavirae*, and again, the only protein shared by all viruses in this kingdom is reverse transcriptase (RT) although most members also share the structural module.[139,146]

The RdRPs and the RTs are two homologous families of enzymes that are sufficiently closely related to each other, especially at the structure comparison level (Fig. 12.2),[100,130,183,261] that they are justifiably considered a single “super-VHP.” RdRP-RT brings together five Baltimore classes of viruses, three within *Orthornavirae* and two within *Pararnavirae*, and given its ubiquity in each of these classes, is the most prominent among all the VHPs.

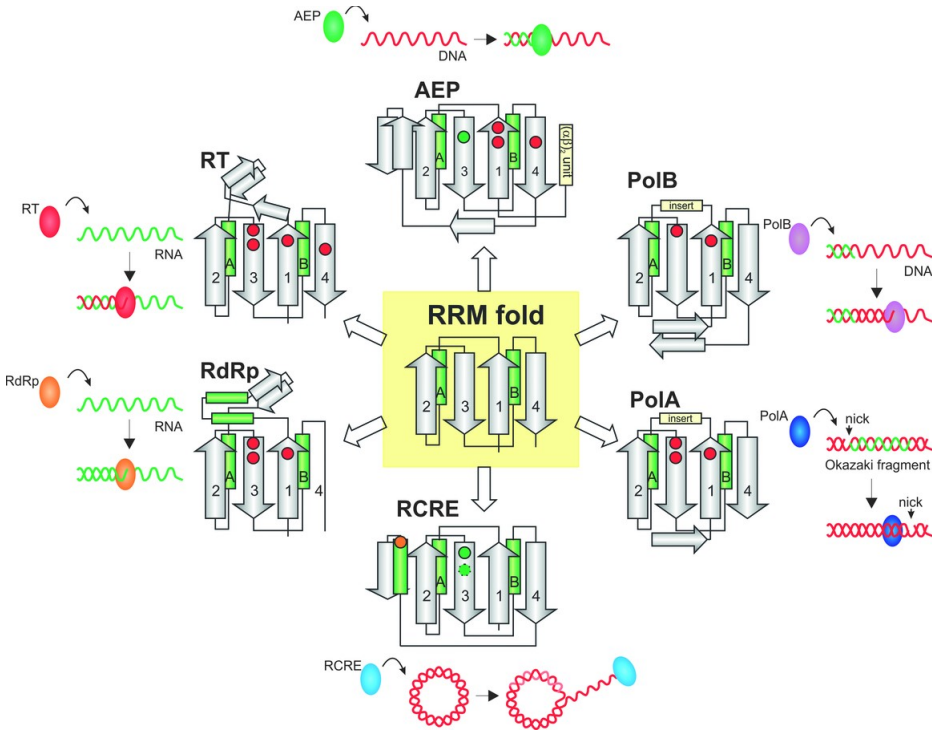

FIGURE 12.2 **Virus hallmark proteins involved in genome replication connect the majority of the virosphere**. Schematic structures of the core domains of the replicative proteins in the four major realms of viruses are shown. The RNA recognition motif (RRM) is one of the most common RNA-binding domains and occurs in all forms of cellular life. Structurally related domains are widespread in many viruses and mobile genetic elements. Replication enzymes containing this module include reverse transcriptase (RT); RNA-dependent RNA polymerase (RdRP); rolling circle replication initiation endonuclease (RCRE); DNA-dependent DNA polymerases of families A (PolA) and B (PolB); and archaeo-eukaryotic primase (AEP). The schematic of the catalyzed reaction is shown for each enzyme, with *red and green wavy lines* representing DNA and RNA strands, respectively. Topologies of the proteins are shown with *arrows* for β-strands and *green rectangles* for α-helices, whereas larger insertions are depicted with *yellow rectangles*. *Red, green, and orange circles* represent catalytic Asp, His, and Tyr residues, respectively. In bacteria, PolA participates in the replication of the lagging DNA strand by connecting Okazaki fragments and in various repair processes, whereas in certain viruses, it functions as the replicative DNA polymerase that was also recruited from a bacteriophage for mitochondrial genome replication in eukaryotes.

RdRP and RT belong to the much broader class of proteins that contain the so-called Palm,[100,130,183] Thumb, and Fingers subdomains and include various polymerases as well as nucleotide cyclases and nucleases (hereafter Palm domain proteins, for brevity). Two families of Palm domain proteins are among the VHPs.

Family B DNA Polymerases

A broad variety of dsDNA viruses in the realms *Varidnaviria* and *Duplodnaviria* encode a DNA-dependent DNA polymerase (DdDP) that is responsible for the virus genome replication. The overwhelming majority of these DdDPs belong to family B (hereafter PolB for brevity), a distinct group of Palm domain proteins (Fig. 12.2) that, apart from the viral polymerases includes the replicative DdDPs of eukaryotes and some groups of archaea.[111,130,209,236] Because, unlike in the case of *Riboviria*, dsDNA viruses have the opportunity to engage the host replication machinery, PolB is not universally present in dsDNA viruses. More specifically, the majority of viruses of the realm *Varidnaviria*, especially those with larger genomes and infecting eukaryotes, encode PolB, but in the realm *Duplodnaviria*, a substantial fraction of viruses lack it.[136,256] Generally, the larger the genome the more replication proteins the virus encodes. All viruses with genomes larger than 140 kb encode DNA polymerases.[112] A distinct subfamily of PolB, the protein-primed DdDPs, is found in several groups of viruses with genomes smaller than 50 kb in the realms *Varidnaviria* (tectiviruses, autolykiviruses, polintoviruses, adenoviruses) and *Duplodnaviria* (phi29-like phages of the family *Salasmaviridae*) as well as in several groups of archaeal viruses (thaspiviruses, halspiviruses, ampullaviruses, ovaliviruses, wyrdviruses) and viruslike DNA plasmids and other Mobile Genetic Elements (MGEs) (eg, casposons, the only group of prokaryotic self-synthesizing transposons currently known).[114,137,140,252]

The small dsDNA viruses that belong to the realm *Monodnaviria* (papillomaviruses and polyomaviruses) as well as the viruses of the realm *Adnaviria* and most of the other dsDNA viruses of hyperthermophilic Thermoproteota (formerly Crenarchaeota) do not encode PolB or any other DdDP (with the exception of ampullaviruses and ovaliviruses). Again, because of the abundance of functionally exchangeable homologs in cellular life forms, the evolutionary history of PolB is far more complex than that of the RdRP-RT. On multiple occasions, viral PolBs apparently were replaced by cellular homologs although protein-primed PolB remains a genuine virus hallmark.[111]

Rolling Circle Replication Initiation Endonuclease

The third VHP with the Palm domain is the endonuclease of the so-called HUH superfamily (after the signature amino acid motif of these nucleases, where U is a hydrophobic amino acid), which is involved in the initiation of DNA replication via the rolling circle mechanism (RCRE, rolling circle replication endonuclease).[34,94,122] The RCRE is universal among the ssDNA viruses in the *Monodnaviria* and is the signature of this virus realm.[127] Some dsDNA viruses in the realms *Adnaviria*, *Duplodnaviria*, and *Varidnaviria* encode RCRE as well.[113] In small dsDNA

viruses of the families *Papillomaviridae* and *Polyomaviridae*, the large protein involved in virus replication (known as T [for tumor] antigen in polyomaviruses) contains a DNA-binding domain that is an inactivated derivative of the RCRE.[58,173]

Palm/RRM Domain, the Core of the Virus Replication Machinery

Apart from the three VHPs discussed above, the Palm domain is represented in another replication VHP, archaeo-eukaryotic primase (AEP; Fig. 12.2) that is encoded by many dsDNA viruses in the realms *Varidnaviria* and *Duplodnaviria*.[94,112] Additionally, some DNA viruses in the realm *Duplodnaviria* and one group of viruses in *Varidnaviria*, the Sputnik-like lavidaviruses (virophages), encode DdDPs of family A (PolA; Fig. 12.2), another group of Palm domain enzymes, that either function in replication or were recruited for a role in transcription.[99,112,119]

The Palm domain is a distinct variety of the RNA Recognition Motif (RRM; Fig. 12.2), one of the most common RNA and DNA binding domains present in a wide variety of proteins in all life forms.[37] Thus, the Palm/RRM domain is the most prominent protein structural unit that connects the replication machineries of all four major realms of viruses as well as all Baltimore classes.[141] Later in this chapter, we discuss the deep implications of this unity for the origin and evolution of viruses.

Helicases and Genome Packaging NTPases

Superfamily 3 Helicases

The most common protein fold in nature is the P-loop domain, the scaffold that harbors the catalytic sites of an enormous diversity of ATPases and GTPases.[157,170,251] Viruses also encode a broad variety of P-loop NTPases, which function in various DNA or RNA transactions during genome replication and/or packaging into capsids, but only three of these qualify as VHPs. By far the most widespread group of P-loop NTPases in viruses is the protein family known as Superfamily 3 helicases (S3H; Fig. 12.3).[78] Notably, S3H is encoded by viruses in different realms and Baltimore classes that widely differ with respect to the genome size and replication mechanisms.[132,141] These include many RNA viruses in the kingdom *Orthornavirae* (primarily, in phylum *Pisuviricota*), the eukaryotic ssDNA viruses in the kingdom *Shotokuvirae* (realm *Monodnaviria*), large DNA viruses that constitute the phylum *Nucleocytoviricota*, and many viruses in the realm *Duplodnaviria*. In all these viruses, the S3H helicases appear to be directly involved in RNA or DNA unwinding during the genome replication. However, in some viruses, for example, parvoviruses, besides its role in genome replication, S3H also functions as a motor that drives the packaging of the viral DNA into preformed empty capsids.[116] Other viruses employ different helicases that are only distantly related to S3H, in the same capacity, as discussed below.

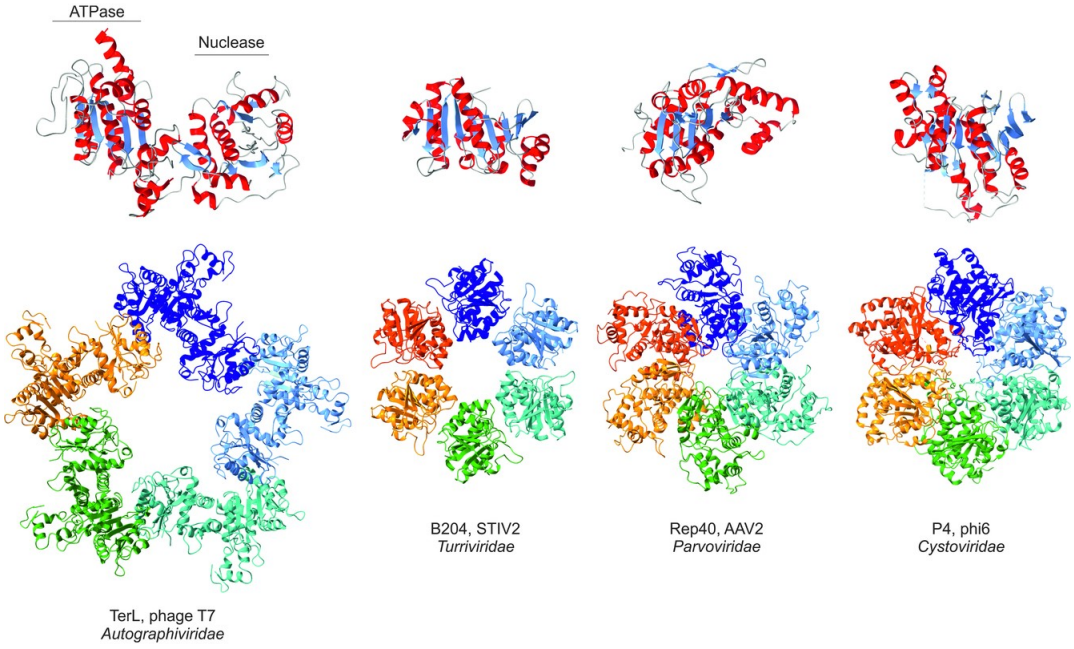

FIGURE 12.3 **Viral helicases and genome packaging ATPases**. The structures of individual proteins and the functional protein oligomers are shown by ribbon diagrams. The structures in the *upper panel* are colored by secondary structure: α-helices, *red*; β-strands, *blue*, whereas in the *bottom panel*, each chain in the corresponding multimeric assemblies is colored differently. PDB ids: T7 TerL, 4bij; STIV2 B204, 4kfu; AAV2 Rep40 monomer, 1s9h; AAV2 Rep68 hexamer, 7jsi; phi6 P4, 5muv. The ATPase and nuclease domains of the TerL are indicated.

Large Terminase Subunit

All dsDNA viruses of the realm *Duplodnaviria* encode another P-loop enzyme, distantly related to SF2 helicases, that contains an N-terminal ATPase and a C-terminal RNase H-like nuclease domain, and is known as the large subunit of the terminase complex (TerL) (Fig. 12.3). The C-terminal nuclease domain of TerL is responsible for the cleavage of virus DNA concatemers formed during replication into genome-sized units, whereas the ATPase domain packages these units into the virus capsid utilizing the energy of ATP hydrolysis.[22,211,262]

FtsK-like Genome Packaging ATPase

Numerous, although not all, dsDNA viruses in the realm *Varidnaviria* encode an ATPase distinct from the large terminase subunit that, however, performs a similar function in DNA packaging into virus capsids (Fig. 12.3). This ATPase belongs to the FtsK family that includes ATPases involved in DNA pumping into daughter cells during bacterial cell division.[101,233] Filamentous ssDNA bacteriophages of the kingdom *Loebvirae* (realm *Monodnaviria*) also encode FtsK-like ATPases which, however, instead of packaging the viral dsDNA into icosahedral capsids, pump the viral ssDNA through the cytoplasmic membrane of the host during virion extrusion.[169,219,232]

RNA viruses typically do not encode genome packaging enzymes, with nucleic acids being coassembled with major structural proteins to form virions.[244] The only currently known exception to this trend are the dsRNA bacteriophages of the family *Cystoviridae* (ream *Riboviria*) that encode packaging enzymes related to the superfamily 4 helicases.[28,56,109,174,175] All packaging enzymes mentioned above form pentameric or hexameric ring-shaped assemblies with a central channel for the translocation of the viral RNA or DNA genome (Fig. 12.3).

Virus Capsid Proteins

Virus capsids show remarkable structural diversity. Compared to proteins involved in genome replication, the sequences of capsid proteins (CPs) evolve much faster, so that the principal approach for the identification of distant relationships among CPs is structure comparison. Altogether, about 20 major CPs with distinct structures have been identified.[138] Importantly, however, the abundances of different capsid shapes and distinct capsid proteins differ widely, so that there are a few common forms and a long tail of rare, odd ones. The majority of viral capsids have icosahedral symmetry, whereas a substantial minority are filamentous or rod-shaped, with helical symmetry. Both icosahedra and helices are thermodynamically favorable forms of protein aggregation, so that both capsid architectures can be built from multiple, unrelated proteins. In particular, at least 10 unrelated protein folds were detected among the major CPs of icosahedral capsids.[138] Among these, only three families consisting of protein components of icosahedral capsids are common enough to qualify as VHPs.

Single Jelly Roll Capsid Protein

The single jelly roll (SJR) CP is the most common of the virus capsid proteins, being the major component of the icosahedral capsids of diverse positive-sense RNA viruses in the kingdom *Orthornavirae* as well as ssDNA and dsDNA viruses in the realm *Monodnaviria* (Fig. 12.4A).[138] Distinct varieties of SJR CP are present also in the dsDNA viruses in the kingdom *Helvetiavirae* (realm *Varidnaviria*),[42,216,222] and as minor capsid proteins, in most other members of the *Varidnaviria*.[60,249]

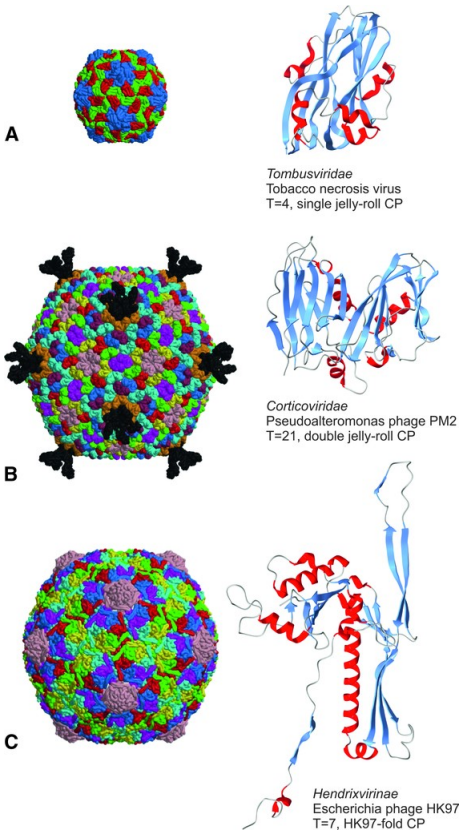

FIGURE 12.4 **Major types of capsid proteins found in icosahedral viruses**. The capsid architectures and ribbon diagrams of the individual capsid proteins are shown. **A**. SJR fold is represented by the CP of tobacco necrosis virus (*Tombusviridae*), PDB id: 1tnv. **B**. DJR fold is represented by the CP of Pseudoalteromonas phage PM2 (*Corticoviridae*), PDB id: 2vvf. **C**. HK97 fold is represented by the CP of *Escherichia* phage HK97, PDB id: 1ohg. The capsid proteins are colored by secondary structure: α-helices, *red*; β-strands, *blue*. (The figures/data were obtained from VIPERdb. (http://viperdb.scripps.edu). VIPERdb v3.0: a structure-based data analytics platform for viral capsids. Montiel-Garcia D, Santoyo-Rivera N, Ho P, Carrillo-Tripp M, Johnson JE, Reddy VS. *Nucleic Acids Research* (2020); doi: 10.1093/nar/gkn1096.)

SJR is a widespread domain in a broad variety of cellular proteins, many of which function by binding carbohydrates, in some cases as multidomain proteins containing SJR fused to a carbohydrate-active enzyme.[138] At least some of the cellular SJR proteins, in particular those of

the tumor necrosis factor (TNF) superfamily, form aggregates resembling virus capsids and thus appear to represent facile material for recruitment as virus CPs.[165] Typically, such recruitment involved acquisition of additional positive charge regions at the protein termini that interact with the viral nucleic acid, promoting its encapsidation.[215] The SJR CPs of eukaryotic RNA and ssDNA viruses, on the one hand, and those of bacterial microviruses (*Monodnaviria*) and halopaniviruses (*Varidnaviria*), on the other hand, show affinity to different groups of cellular SJR proteins.[138] Thus, SJR CPs apparently have been recruited by viruses from cellular ancestral proteins on at least two and likely more independent occasions.

Double Jelly Roll Capsid Proteins

The CPs of the diverse dsDNA viruses in the kingdom *Bamfordvirae* of the realm *Varidnaviria* consist of two fused jelly roll domains and are known as double jell roll (DJR) CPs (Fig. 12.4B).[4,135] The second kingdom currently included in *Varidnaviria*, *Helvetiavirae*, consists of three families of bacterial and archaeal viruses, *Sphaerolipoviridae*, *Simuloviridae*, and *Matshushitaviridae*, that possess two SJR CPs encoded in separate genes.[42,216,222] The SJR CPs of *Helvetiavirae* form homo- and heterodimers, which further aggregate into pseudohexagonal, heterohexameric capsomers that structurally resemble the homotrimeric capsomers of DJR-CPs of the *Bamfordvirae*. Given this structural similarity, it has been hypothesized that DJR-CPs evolved via fusion of the two SJR-CPs following a gene duplication.[213] However, subsequent focused search for cellular homologs of the DJR-CP identified several families of such proteins, in some of which the DJR domain is fused to various carbohydrate-active enzymes.[144] The jelly roll domains of the DJR CPs of *Bamfordvirae* show closer structural similarity to some of these cellular proteins than to the SJR-CPs of *Helvetiavirae*, suggesting that the capsid proteins in the two kingdoms of *Varidnaviria* were acquired independently from different cellular ancestors.[144]

HK97 Capsid Proteins

The third capsid protein VHP is the CP of the tailed icosahedral viruses of the realm *Duplodnaviria*,[4,49,234] which has a characteristic HK97-like fold, after the phage for which the capsid structure was solved first.[258] No cellular homologs of the HK97 CP with the same domain architecture have been identified (except for nanocompartments known as encapsulins that apparently were derived from the HK97 CP), but a more complex relationship with two families of cellular proteins has been discovered. Apparently, the HK97 CP evolved from bacterial proteins known as dodecins, via insertion of an uncharacterized domain (Fig. 12.4C).[92,142] Dodecins are involved in flavin storage in bacteria and form dodecameric spherical structures that can be considered (mini)capsidlike.[82]

Evolutionary Roots of the Virus Hallmark Proteins and Implications for the Origin of Viruses

The observations on the origins of the two functional classes of VHPs, those involved in virus genome replication and those forming virus structure or facilitating its formation like packaging ATPases (replication and structural VHPs, for brevity), suggest different routes of evolution and translate into a parsimonious scenario for the origin of viruses. The replication VHPs, particularly RdRP and RT but also protein-primed DNAP, RCRE, and S3H, have only distant homologs among cellular proteins, suggestive of an early origin, likely antedating the Last Universal Cellular Ancestor (LUCA). Evolutionary reconstructions suggest that the LUCA was a population of organisms comparable in complexity with the extant prokaryotes.[69,118,142,180,191,199] In particular, multiple polymerases and helicases were apparently already represented in the LUCA.[66,130,156] Furthermore, RdRP and RT are the types of replicative enzymes that can be expected to exist at the time of the transition from the hypothetical primordial RNA-protein world to the DNA-RNA-protein life of the modern variety.[65,90] Looking even deeper into the earliest stages of evolution, it seems likely that the currently ubiquitous RRM was one of the first protein domains to become a component of the replication system in the hypothetical RNA world,[141] conceivably, as a cofactor to ribozyme polymerases. The subsequent diversification of the RRM including the emergence of catalytic sites gave rise both to the replication VHPs and to cellular polymerases.

The origins of the structural VHPs appear to be quite different. For each of these, in particular, each of the three widespread CPs that form icosahedral capsids, as well as the packaging ATPases, specific cellular ancestors could be identified.[138] The respective families of cellular proteins are present and highly diversified in all three domains of life, suggesting that they have already emerged by the time of the LUCA. Thus, the structural VHPs, most likely, evolved from cellular ancestors early in the evolution of life, but already after the advent of modern-like protocells.[141] For other structural components of virions, later origin from cellular ancestors was inferred.

Taken together, the findings on the origins of the replicative and structural VHPs suggest a mixed scenario for the origin of viruses (Fig. 12.5).[141] The replication machineries of viruses appear to originate from the primordial pool of replicators in which different forms of RNA and DNA genomes that subsequently gave rise to different Baltimore classes of viruses have already evolved. The replication machineries of viruses, particularly those with DNA genomes, were substantially reshaped by recruitment of components from the hosts, in some cases with replacement of ancestral genes. Nevertheless, the cores of virus replication systems seem to antedate modern cells. In terms of the traditionally discussed hypotheses on the origin of viruses, this part of the scenario corresponds to the “virus early” hypothesis. In contrast, the structural VHPs clearly derive from cellular ancestors and were recruited for functions in the virions at different stages of evolution, which corresponds to the “escaped genes” hypothesis of virus origin. This mixed, “chimeric” scenario appears to be the most parsimonious explanation of the origin of viruses, and remarkably, it is recapitulated at later stages of the evolution of many viruses. In particular, various groups of ssDNA viruses in the realm *Monodnaviria* appear to have evolved independently when a small RCR plasmid merged with the gene encoding the SJR CP of a positive-sense RNA virus.[113,134]

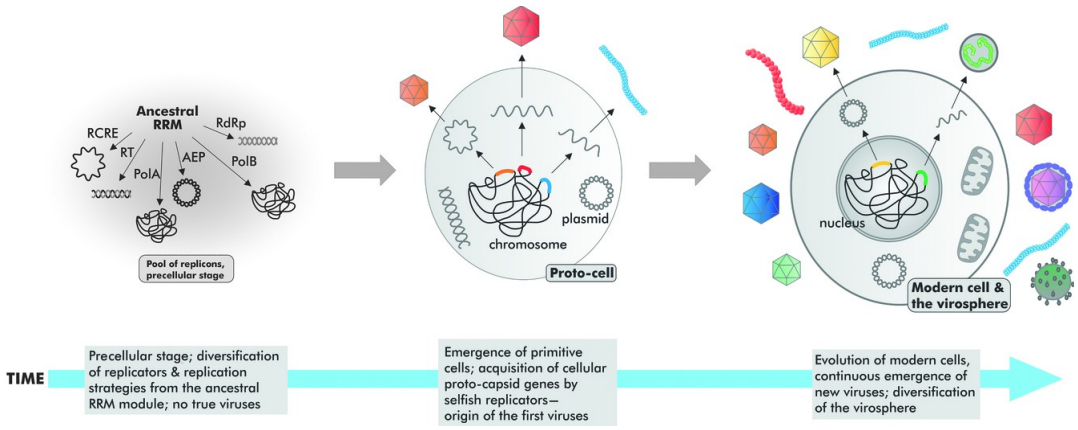

FIGURE 12.5 **The chimeric scenario for the origin of viruses**. (Modified with permission from Krupovic M, Koonin EV. Multiple origins of viral capsid proteins from cellular ancestors. *Proc Natl Acad Sci U S A*. 2017;114(12):E2401-E2410.)

The first virus genomes to emerge, most likely, encoded only a replication VHP coming from a primordial replicator and a structural VHP captured from a host cell. One of such prototypical combinations would have been RdRP-SJR-CP, an arrangement that is found in some of the simplest extant RNA viruses, such as totiviruses. Subsequent evolution of most virus lineages involved accretion of proteins through recruitment of host genes as outlined in the next sections.

Moderately Conserved Proteins Involved in Core Virus Functions

Beneath the VHPs in the hierarchy of virus proteins by evolutionary conservation is a larger collection of proteins that are conserved in a substantial but limited diversity of viruses within a kingdom (more typically, within a phylum) and participate in core functions, such as genome replication and expression. Most viruses encode such additional core proteins, with the exception of those with the smallest genomes and simplest genome organization, such as several groups of RNA viruses in the kingdom *Orthornavirae*, for example, toti-like and tombus-like viruses, and many ssDNA viruses in the realm *Monodnaviria*, for example, circoviruses and parvoviruses, in which the protein repertoire is limited to VHPs. For most of these moderately conserved proteins, origin via recruitment of a host gene is readily traceable.

Viruses vary enormously in the extent to which core functions, such as genome replication and expression, rely on virus-encoded proteins or are relegated to directly recruited host proteins. The "logic" of the labor separation between virus and host proteins appears traceable in some cases but not others. In particular, among RNA viruses, the polymerases responsible for genome replication are typically encoded in the virus genome because RNA replication does not normally occur in uninfected cells. Otherwise, however, these viruses substantially differ in the extent of their replicative autonomy from the host, with those with the smallest genomes encoding no additional proteins for core functions, but those with larger genomes accruing several such proteins. The small genomes of the viruses in the realm *Monodnaviria* accommodate a single replicative protein, otherwise relying on the cellular machinery. The dsDNA viruses with larger genomes, in the realms *Varidnaviria*, *Duplodnaviria*, and *Adnaviria*, demonstrate striking diversity. In particular, most of the large and giant viruses within *Nucleocytovirecetes* encode (nearly) complete replication and expression systems, which can be construed as an essential adaptation for the autonomous replication of these viruses within "virus factories" in the cytoplasm of infected cells.[126] However, some members of *Nucleocytovirecetes* underwent different degrees of reductive evolution that accompanied relocation of the virus replication and expression site to the cell nucleus,[248] and involved the loss of many or even most of the viral genes involved in these core processes. Some of the smaller viruses within *Varidnaviria* that reproduce in prokaryotic cells or in the eukaryotic nuclei encode DNA polymerases but otherwise primarily rely on host proteins for the genome replication and expression. Among the dsDNA viruses in the realm *Duplodnaviria*, the variation is even greater as some of these viruses encode virtually autonomous replication and transcription systems,[112,206] whereas others have virtually no proteins involved in these processes.[256] More specifically, most of the dsDNA viruses of hyperthermophilic Thermoproteota, in particular, those in the realm *Adnaviria*, apparently rely on the host replication and expression apparatus (almost) completely.[147]

There are several recurrent structural and functional themes among the moderately conserved core virus proteins. It is unrealistic to cover in this chapter all or even most of these proteins, but we discuss the most common and characteristic ones.

P-loop Enzymes

The P-loop fold is the most common one among all protein domains.[170] The small set of VHPs discussed above includes three P-loop enzymes, SF3 helicases, and two packaging ATPases. In addition, many viruses encode a considerable diversity of P-loop enzymes with a more narrow spread. The most prominent of these are several groups of helicases, some of which are signatures of virus phyla or classes. Apart from the super-VHP, the SF3 helicase, SF1 helicases are represented in a broad variety of positive-sense RNA viruses in the phylum *Kitrinoviricota* and in the order *Nidovirales* of the phylum *Pisuviricota*, whereas SF2 helicases are encoded by two large virus families (*Flaviviridae* and *Potyviridae*) among the *Pisuviricota*, in which most viruses possess S3H.[260] Among the positive-sense RNA viruses, the presence of a helicase strongly correlates with the genome size: all viruses with genomes in excess of about 6 kb encode a helicase, whereas those with smaller genomes do not. This pattern implies that, among the positive-sense RNA viruses, helicases are required for efficient replication of long genomic RNA and were independently acquired on at least four separate occasions, by recruitment of host enzymes or, in some cases, by intervirus recombination. This is a striking case of convergent acquisition by viruses of genes with similar functions (but not necessarily closely related or homologous at all) driven by common selective forces, which may be considered one of the key principles of virus evolution.

SF2 helicases are also encoded by most of the large DNA viruses of the class *Nucleocytovirecetes* in the realm *Varidnaviria* as well as by herpesviruses and numerous tailed bacteriophages in the realm *Duplodnaviria*.[112] Members of *Nucleocytovirecetes* encode up to four distinct SF2 helicases (eg, in poxviruses) that appear to have been independently captured from the hosts and function in virus genome expression and other, still not fully understood roles. Bacterial viruses in the realm *Duplodnaviria* encode the DnaB-like SF4 helicase homologous to the

bacterial replicative helicases that in many viruses is fused to a bacterial DnaG-like primase domain. This architecture of the virus primase-helicase mimics the fusion of S3H and AEP that is a signature of *Nucleocytovirecetes* and is also found in some viruses in *Duplodnaviria* as well as plasmids. Actually, nearly all possible combinations of SF1-SF4 helicases with AEP and DnaG-like primases can be found among dsDNA viruses.[112] In some cases, the fusion proteins even contain family A or family B DNA polymerases. Once again, in different viruses, selection for efficient replication apparently drove the evolution of the composite replicative proteins from distinct but functionally analogous domains. Many bacterial and archaeal viruses within *Duplodnaviria* also encode a DNA-dependent ATPase or helicase of the AAA+ superfamily that is involved in the initiation of virus DNA replication.[168,256] It should be noticed, however, that replicative helicases and ATPases are missing in many groups of DNA viruses, among both *Varidnaviria* and *Duplodnaviria*, conceivably, because host proteins with the corresponding activities are directly recruited to participate in virus genome replication.

Apart from the helicases, a class of P-loop enzymes common among dsDNA viruses with large DNA genomes, in both *Varidnaviria* and *Duplodnaviria*, are nucleotide and nucleoside kinases, in particular, thymidine and thymidylate kinases. These kinases are common but not ubiquitous among *Nucleocytovirecetes* as well as among *Herpesvirales* and *Caudoviricetes*.[72,168,179,266] This is another case of convergent capture of enzymes with the same functionality by multiple lineages of viruses under evolutionary pressure to complement the host nucleotide metabolism that, however, manifests only in certain hosts and cell types.

Proteases

In a broad variety of viruses, all or some of the mature virus proteins are produced by proteolytic processing of precursors. In many families of positive-sense RNA viruses in the phyla *Kitrinoviricota* and *Pisuviricota*, either the entire genomic RNA or its large portion encoding nonstructural proteins are expressed as a polyprotein that contains one or more protease domains.[260] These viruses encode proteases of two expansive families with unrelated folds, papainlike and chymotrypsinlike (MEROPS clans PA and CA, respectively),[75,77] and some viruses encompass both types of proteases (Fig. 12.6). A unique feature of RNA virus chymotrypsinlike proteases is the replacement of the catalytic serine by a cysteine in most of them, such as the well-studied proteases of picornaviruses.[75] Another remarkable twist is the recruitment of a chymotrypsinlike protease as the capsid protein replacing the ancestral SJR CP in alphaviruses within *Togaviridae*, an expansive family of animal positive-sense RNA viruses in the phylum *Kitrinoviricota*. Most likely, alphaviruses acquired the protease from another RNA virus family, *Flaviviridae*,[138] in which this protein is not a virion component but, rather, performs a function typical of proteases, namely, polyprotein processing.[115] Convergently with the case of the SJR CPs, recruitment of the protease as the alphavirus CP involved acquisition of a positively charged, nonstructured N-terminal region.[178] The reverse-transcribing RNA viruses of the kingdom *Pararnavirae* also produce polyproteins that are cleaved by a virus-encoded aspartate protease (Fig. 12.6).[228]

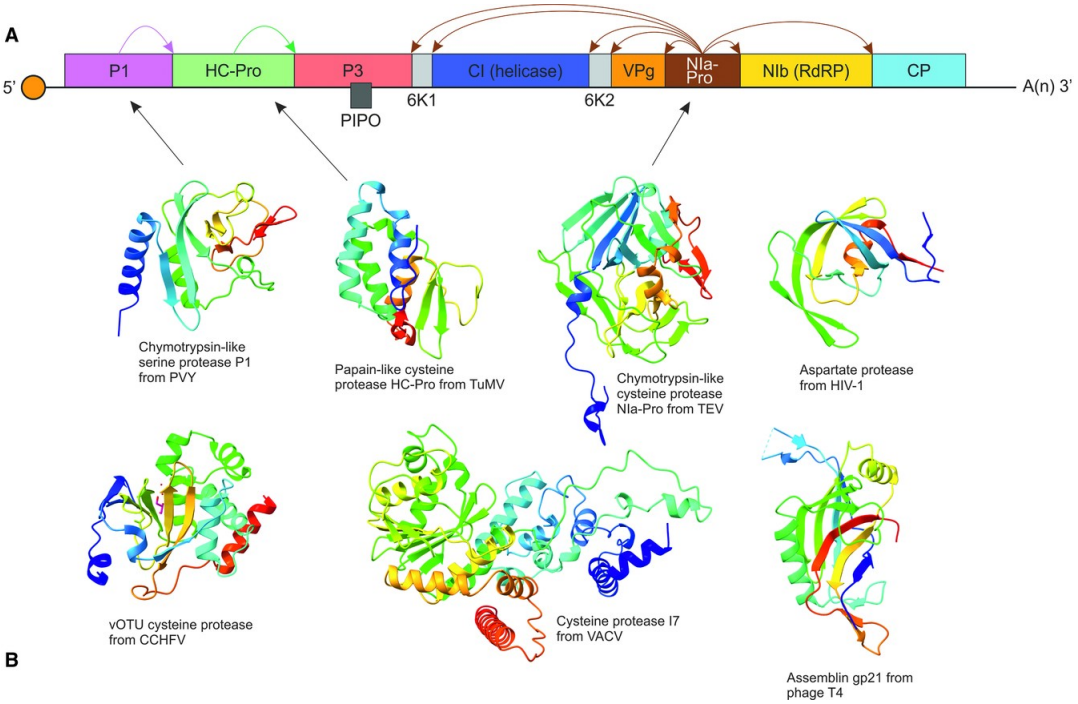

FIGURE 12.6 Proteases encoded by RNA and DNA viruses. **A**. Proteolytic processing of the polyprotein encoded by potato virus Y (family *Potyviridae*). Cleavage sites of the three virus-encoded proteases are indicated by *bent arrows* colored using the same colors as the genes encoding the corresponding proteases. **B**. Ribbon diagrams of the structures of serine, cysteine, and aspartate proteases encoded by RNA and DNA viruses, including the three proteases of potyviruses: P1 (AlphaFold2 model), HC-Pro (PDB id: 3rnv), and NIa-Pro (PDB id: 1lvm); aspartate protease of retroviruses (PDB id: 2i4d); vOTU deubiquitinase of nairoviruses (PDB id: 3phu); virion maturation protease I7 of poxviruses (RoseTTAFold model); capsid maturation protease of duplodnaviruses (PDB id: 5jbl). The structures are colored using the rainbow scheme from blue N-terminus to red C-terminus. Abbreviations: HC-Pro, helper component-protease; CI, cylindrical inclusion protein; VPg, viral protein genome-linked; NIa-Pro, nuclear inclusion a-protease; CP, capsid protein; PIPO, pretty interesting *Potyviridae* ORF; PVY, potato virus Y; TuMV, turnip mosaic virus; TEV, tobacco etch virus; HIV-1, human immunodeficiency virus 1; CCHFV, Crimean-Congo hemorrhagic fever virus; VACV, vaccinia virus.

The utility of proteases for RNA viruses of eukaryotes is obviously linked to the features of the eukaryotic translation whereby the great majority of mRNAs are monocistronic (that is, contain a unique initiation site near the 5′-terminus from which a single protein is translated). Thus, to

produce individual, mature proteins, RNA viruses employ three strategies, some viruses combining two or even all three: proteolytic processing of precursor polyproteins, transcription of subgenomic mRNAs, and genome segmentation. Under the selective pressure for expressing individual proteins, viruses within *Riboviria* acquired unrelated proteases on at least three independent occasions (the papainlike, chymotrypsinlike, and aspartate proteases). Most likely, however, there have been more of such recruitment events because both the papainlike and chymotrypsinlike proteases in different groups of positive-sense RNA viruses are only distantly related to each other (Fig. 12.6). Furtherrmore, some (−)RNA bunyaviruses, such as members of the *Nairoviridae* and *Phenuiviridae* families, and (+)RNA viruses of the orders *Tymovirales* and *Nidovirales* encode homologs of the ovarian tumor protease, a papainlike cysteine protease, which is involved in deubiquitination of various host proteins, thereby promoting evasion of the innate immune response.[18,44,53,207]

For some of the RNA virus proteases, the origin from specific families of cellular proteases is traceable. The chymotrypsinlike proteases in the order *Picornavirales* were apparently derived from the HtrA family of bacterial proteases.[133] The aspartate protease of the reverse-transcribing viruses seems to originate from the Ddi1 protease, a highly conserved component of the eukaryotic ubiquitin signaling network.[148,230]

Although viruses with DNA genomes typically do not encode polyproteins, most of the viruses of eukaryotes in the realm *Varidnaviria* encode a cysteine protease (MEROPS clan CE), distantly related to papainlike proteases (clan CA) that is required for the maturation cleavage of capsid proteins.[11,24,29,160,255] Notably, this protease is specifically related to the eukaryotic deubiquitinating enzyme (DUB) Ulp1 that probably was recruited by varidnaviruses at an early stage of their evolution in eukaryotes.[137] The majority of the dsDNA viruses in the realm *Duplodnaviria* encode an unrelated serine protease with a unique fold (MEROPS clan SH; Fig. 12.6), known as assemblin in herpesviruses, that also catalyzes the proteolytic maturation of the major capsid protein or cleavage of the scaffolding protein.[35,50,242,272] Remarkably, assemblin protease is structurally related to proteins forming the tubes of phage tails,[64] suggesting that the iconic tails of *Caudoviricetes* evolved from a protease, mirroring the evolution of alphaviral CPs from a polyprotein-processing protease and, more broadly, supporting the exaptation scenario where viral structural proteins have evolved through refunctionalization of preexisting cellular and viral proteins. Notably, in some tailed phages, assemblin is replaced by a different serine protease related to bacterial ClpP.[164]

Sporadically, other proteases, such as metalloproteases of the zincin family, are also encoded in phage genomes.[269] Altogether, the diversity and complex evolutionary history of virus proteases further demonstrate the pervasive trend of convergent capture of unrelated host proteins with similar functions by different virus lineages.

DNA-Dependent RNA Polymerases and Transcription Factors

Most of the dsDNA viruses with large genomes within the classes *Nucleocytovirecetes*, *Caudoviricetes*, and *Naldaviricetes* (ie, Baculo-like viruses) encode their own transcription machinery. The DNA-dependent RNA polymerase (RNAP) subunits and core transcription factors encoded by the members of *Nucleocytovirecetes* and *Naldaviricetes* have readily identifiable homologous counterparts among the corresponding components of the eukaryotic transcription machinery (Fig. 12.7A).[27,83,91,171,200] However, the evolutionary history of the RNAPs appears to be highly complex, with multiple gene exchanges and displacements between the viruses and their eukaryote hosts.[84,266]

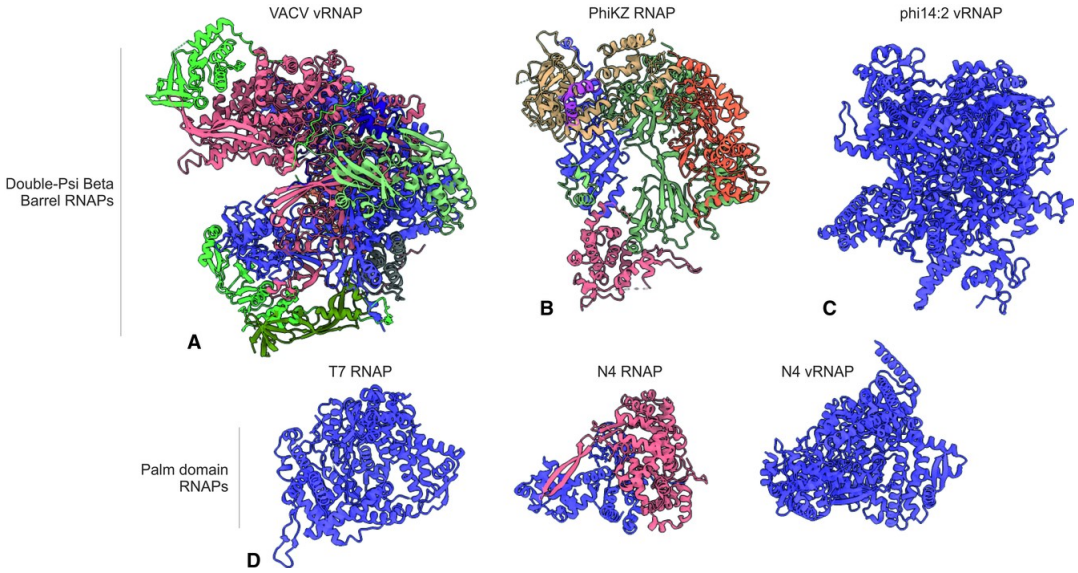

FIGURE 12.7 **Viral DNA-dependent RNA polymerases (RNAPs)**. **A-C**. Double Psi Beta Barrel RNAPs. **A**. Multisubunit RNAP of vaccinia virus (VACV). PDB id: 6ric. **B**. Multisubunit RNAP of *Pseudomonas* bacteriophage PhiKZ. PDB id: 7ogr. **C**. Single-subunit RNAP of *Cellulophaga* bacteriophage phi14:2. PDB id: 6vr4. **D**. Palm domain RNAPs of bacteriophages T7 and N4. Note that RNAP of N4 (*middle*; PDB id: 6dt7) is a heterodimer, whereas T7 RNAP (PDB id: 1h38) and N4 vRNAP (PDB id:2po4) are single-subunit enzymes. Prefix "v" in vRNAP indicates that the RNAP is encapsidated into virions. Each chain is colored differently in the corresponding structures to highlight the difference between single-subunit and multisubunit enzymes.

The diversity of the transcription machinery in tailed bacteriophages is far greater. Different members of *Caudovirecetes* encode two unrelated types of RNAPs. Numerous groups of bacteriophages have homologs of the highly conserved, multisubunit bacterial RNAP in which the Double-Psi Beta Barrel (DPBB) domains from the two large subunits jointly constitute the catalytic site (Fig. 12.7B).[43,231] However, the phage versions are often highly derived, demonstrating extreme divergence as well as subunit fusion and loss of individual subunits.[48] In some phages, the RNAP is reduced to a single protein containing two DPBB domains (Fig. 12.7C). However, several groups within *Caudovirecetes*, in particular, T7-like phages, instead of the DPBB-domain RNAP, encode a single-subunit RNAP containing a Palm domain and derived from the A family

DNAPs (Fig. 12.7D).[103,237] As in the case of DPBB RNAPs, some T7-like RNAPs are partitioned into several subunits.[182] Notably, this RNAP was recruited for mitochondrial genome transcription in eukaryotes, most likely, from a prophage that was integrated into the genome of the pre-mitochondrial endosymbiont.[63,87,227] The driving forces behind the enormous diversification of the RNAPs in tailed phages remain a puzzle. Some of the phage RNAPs, from both the DPBB and the Palm domain varieties, are encapsidated into virions and are injected into the host cell along with the genome to transcribe the phage early genes.[48,188] The structural requirements for such virion RNAPs are likely to be substantially different from those for the RNAP involved in bacterial transcription, resulting in dramatic changes in the phage RNAP sequence, structure, and subunit organization.

Capping Enzymes

Some common components of the virus proteomes can be naturally linked to the key molecular features of the hosts. Nearly all eukaryote mRNAs contain a 5′-terminal cap that is required for translation initiation and protects the mRNA from degradation.[210] Some positive-sense RNA viruses, in particular, those in the orders *Picornavirales* and *Potatovirales* within *Pisuviricota*, circumvent the requirement for the cap by protecting the RNA with a 5′terminal, covalently bound virus protein and initiating translation at distinct RNA domains, known as internal ribosomal entry sites (IRES).[74,104] Alternatively, some RNA viruses, such as hepaciviruses, pestiviruses, and pegiviruses (family *Flaviviridae*), lack both the cap and the terminal protein, and instead only carry an IRES, which enables efficient translation of mRNAs lacking a 5′ cap structure.[229] However, the majority of the viruses infecting eukaryotes make capped mRNAs via one of three strategies. Segmented negative-sense RNA viruses (orders *Bunyavirales* and *Articulavirales* within *Negarnaviricota*) encode a distinct endonuclease that clips off cap-containing 5′-terminal segments of host mRNAs to initiate the synthesis of capped virus mRNAs.[198] Numerous other viruses, primarily, those that (at least, for part of their lifecycle) reproduce inside the nucleus, in particular, all reverse-transcribing viruses in the kingdom *Pararnavirae*, all small DNA viruses in the realm *Monodnaviria*, smaller viruses within *Varidnaviria* (eg, adenoviruses) and herpesviruses, exploit the host capping apparatus.[45] However, viruses reproducing in the host cytoplasm, in particular, the majority of the eukaryote-infecting RNA viruses within *Orthornavirae* and most of the *Nucleocytoviricetes*, encode their own capping enzymes that apparently evolved from the host enzymes on several independent occasions and show complex evolutionary relationships.[125] Some of these enzymes are divergent homologs of the host capping machinery, as in the case of *Nucleocytoviricetes*, which encode the enzymatic domains with RNA 5′-triphosphatase, RNA guanylyltransferase, RNA (guanine-N7)-methyltransferase, and 2′-*O*-methyltransferase activities catalyzing all the steps of cap formation, either as separate proteins or as protein fusions encompassing two or more domains.[150,226] In dsRNA reoviruses, the capping enzyme is part of the virion and contains four enzymatic domains, which sequentially catalyze all four steps of the cap synthesis.[214] Coronaviruses and likely other members of the *Nidovirales* also use the canonical capping pathway and encode the enzymes for all four reactions.[25,45] The RNA guanylyltransferase encoded by these viruses has evaded detection until recently but now has been demonstrated to reside in the NiRAN domain of the RdRP.[155,250]

The enzymes responsible for different reactions required for capping can be nonhomologous from one group of viruses to the next. For instance, RNA 5′-triphosphatase, which catalyzes the cleavage of the phosphate bond in 5′-triphosphorylated mRNA, can belong to four different enzyme families,[45] indicative of multiple independent gene acquisitions and/or replacements. Whereas members of the *Nucleocytoviricetes* and reoviruses synthesize their caps using the canonical pathway that also operates in eukaryotes, many RNA viruses evolved unconventional pathways for synthesizing the cap,[45] which, however, has the same structure as that of the hosts (Fig. 12.8). The first of such distinct capping pathways is used by negative-sense RNA viruses of the order *Mononegavirales*.[197] The L protein of mononegaviruses is a multifunctional enzyme responsible for both RNA replication and capping. One of the L subdomains has the GDP polyribonucleotidyl transferase (PRNTase) activity and transfers GDP, rather than GMP, as it occurs in the canonical pathway, to the RNA 5′ end.[197,208] The methyltransferase subdomain of the L protein then methylates both ribose-2′-O position of the first nucleotide, and subsequently, the guanine-N7 position, thereby generating the mature cap structure (Fig. 12.8). Alphaviruses and nodaviruses use a different capping pathway by virtue of a unique bifunctional enzyme with the methyltransferase-guanylyltransferase (MTase-GTase) activity.[6,7] Unlike cellular cap methyltransferases, which methylate GTP only after it is transferred to the 5′ end of the mRNA, the alphaviral MTase-GTase first methylates GTP and only subsequently conjugates it to the 5′ end of the viral mRNA (Fig. 12.8). Furthermore, the first step of the alphavirus/nodavirus capping reaction, namely, dephosphorylation of the triphosphorylated RNA 5′ end, which is performed by a dedicated RNA 5′-triphosphatase in the canonical pathway, is carried out by the viral helicase, using the same NTPase active site that powers the RNA unwinding activity.[7] Remarkably, the MTase-GTase of alphaviruses is located at the neck of membrane-bound replicase complexes, also known as spherules, where it forms a dodecameric, crown-shaped ring, which acts as a gateway between the lumen of virus replication centers and the host cytoplasm.[106,271] The unique MTase-GTase of alphaviruses evolved through relatively minor structural elaborations of the canonical SAM-dependent MTase fold,[106] a testament to the enormous “creativity” of virus evolution, likely stimulated by the prohibitive genome size constraints.

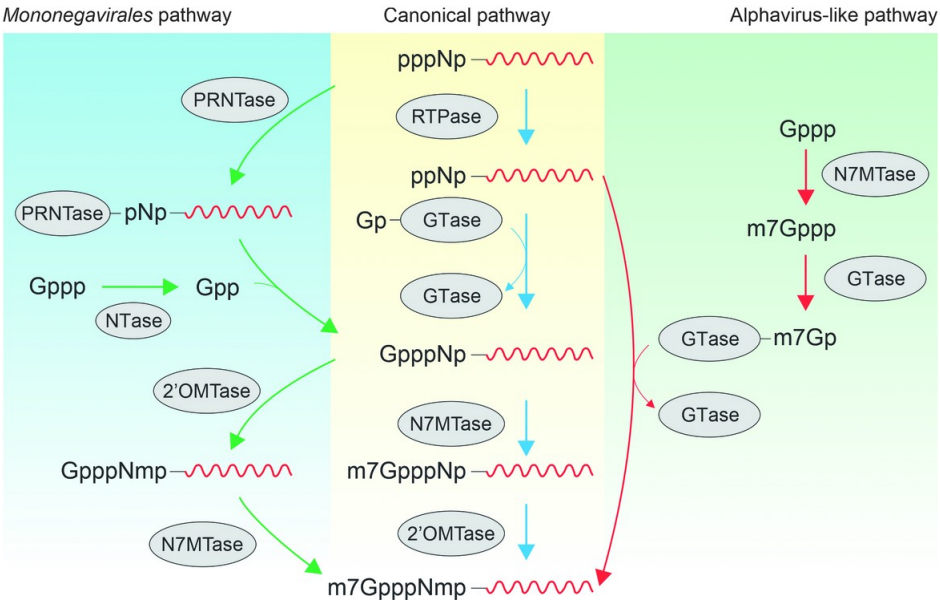

FIGURE 12.8 **Diversity of cap formation pathways encoded by viruses**.

Lineage-Specific Virus Proteins Involved in Core Processes

Apart from the common contributors to the core processes of genome replication and expression as well as morphogenesis, some groups of viruses, including even viruses with larger RNA genomes, encode proteins dedicated to more specific roles in such processes. Notable examples of such specialized proteins contributing to genome replication of RNA viruses are the exonuclease (ExoN) and a pyrimidine-specific endonuclease (NendoU) that are conserved in all viruses of the order *Nidovirales* within *Pisuviricota* but not found in any other viruses.[76,245] Distant homologs of both nucleases were identified in eukaryotes indicating that these genes were recruited from the host at the onset of the evolution of *Nidovirales*. Both enzymes are required for coronavirus RNA replication, where ExoN supplies the proofreading activity which, along with other factors, apparently provides for genome expansion.[61,76] Another group of positive-sense RNA viruses with relatively large genomes, members of the family *Closteroviridae* within *Kitrinoviricota*, encode a molecular chaperone of the HSP70 family that is required for virion assembly.[10,15,47]

Viruses with large dsDNA genomes in the realms *Varidnaviria* and *Duplodnaviria* harbor a vast repertoire of lineage-specific proteins involved in genome replication and expression as well as virion morphogenesis. As an example, it is interesting to mention that viruses from several families within *Nucleocytoviricotes* encode histones.[59,167,218,241,246,264] For instance, members of *Marseilleviridae* encode unique histone doublets, which form nucleosomes remarkably similar to those found in all eukaryotes.[167,246] Certain members of *Phycodnaviridae*, another family within *Nucleocytoviricotes*, instead encode a different chromatin protein of the DVNP family (PFAM: PF19060.3), exemplified by EsV-1-52 from Ectocarpus siliculosus virus 1, that promotes genome compaction and replaced the histones in dinoflagellates, a group of single-celled eukaryotes.[79,97]

Virus Proteins Involved in Virus-Host Interactions

All viruses are entangled in diverse interactions with the host organisms, in particular, coping with the multiple lines of defense, including innate and, in some groups of organisms, adaptive immunity.[5] A key aspect of virus evolution is the perennial arms race with the host whereby the hosts evolve and adapt defense mechanism, whereas viruses escape through the fixation of resistance mutations and evolution of antidefense mechanisms.[67] Thus, both defense and antidefense systems tend to evolve rapidly, in terms of gene gain and loss as well as sequence and structure change. As can be expected, the repertoires of dedicated proteins involved in virus-host interactions are tightly linked to the host biology. In particular, viruses of prokaryotes and eukaryotes have completely different sets of such proteins, and so do viruses of unicellular eukaryotes, plants, and animals.

Viruses with large DNA genomes in the realms *Varidnaviria* and *Duplodnaviria* encode numerous proteins involved in various types of virus-host interactions, mostly, counter-defense. As a rule of thumb, about half of the proteins in large virus genomes (those in excess of about 100 kb) have dedicated functions in virus-host interactions, as observed, for example, in such thoroughly characterized group as chordopoxviruses within *Nucleocytovirecetes*.

Viruses with small genomes in the realms *Riboviria* and *Monodnaviria* encode few proteins that function solely in virus-host interaction. Typical of these viruses is gene sharing whereby virus proteins responsible for core functions moonlight in virus-host interactions. For example, most viruses in the family *Picornaviridae*, in addition to the main chymotrypsinlike protease responsible for most of the polyprotein cleavages (see above) encompass additional proteases, chymotrypsinlike 2A, which is encoded directly downstream of the capsid proteins, or papainlike L(leader), the N-terminal portion of the respective polyproteins. These proteases are essential for virus reproduction because they cleave the polyprotein *in cis*, but their unique function is the cleavage of the host translation initiation factor eIF4G and some other host proteins, inhibiting translation of the host capped mRNAs and suppressing innate immunity, including apoptosis.[5,39,184] Notably, in other picornaviruses, proteins occupying the same positions in the polyprotein but lacking protease activity inhibit host translation via other, nonenzymatic mechanisms. Apart from these "security proteins," the 3A protein of picornaviruses that is responsible for the membrane localization of the virus replication complex has been shown to inhibit apoptosis.[5,102,117]

A heterogeneous set of positive-sense RNA viruses in both *Kitrinoviricota* and *Pisuviricota*, including alphaviruses, rubella virus, hepeviruses,

and some coronaviruses, encode, within their nonstructural polyproteins, the Macro domain (so named after the macro variant of histone H2A that contains this domain).[121] In addition, Macro domains are encoded by some of the *Nucleocytovirecetes* as well some bacteriophages within *Duplodnaviria*. The Macro domains in RNA and DNA viruses are distantly related and apparently were acquired from the host on multiple, independent occasions. Macro domains are phosphohydrolases that are specific for (poly)ADP-ribose.[195] The primary function of the Macro domain is likely to be in antidefense, via the reversal of ADP-ribosylation of virus proteins, given that poly ADP-ribose polymerases are known antivirus factors.[13,55,159] However, direct involvement in virus genome replication cannot be ruled out either, especially, in betacoronaviruses where the nsP3 protein contains two derived Macro domains and binds RNA.[161,162] The macrodomain is a striking case of recruitment of a highly specific enzyme by unrelated viruses infecting diverse hosts, illustrating both the wide spread of some defense mechanisms and the multifunctionality of virus proteins.

However, even some of the viruses with the smallest genomes encode dedicated proteins that contribute to virus-host interaction in a specific manner dictated by the host biology. Viruses of plants, all of which have comparatively small genomes, apparently, because large viruses cannot spread between plant cells, stand out with respect to the prominence of such links between dedicated virus proteins and biological features of the hosts. A prominent case in point are the movement proteins (MP) of plant viruses that enable the movement of viral RNA or DNA among the plant cells through plasmodesmata.[172,238] A great variety of both RNA viruses from *Orthornaviria* and *Pararnaviria*, and ssDNA viruses infecting plants encode homologous MPs of the so-called 30K family that bind virus RNA or DNA and transport it from cell to cell.[177,189] Undoubtedly, the MPs extensively spread horizontally under strong selection because intercellular movement is a condition of virus spread in plants. The origin of the 30K family of MPs remains obscure. Several groups of positive-sense plant RNA viruses within *Kitrinoviricota* lack a 30K family MP but instead encode three unrelated proteins involved in virus movement, one of which is a distinct SF1 helicase, in the so-called triple gene block.[185,190] Other plant viruses employ unrelated proteins as MPs. It appears likely that additional MPs will be discovered in those plant viruses that lack both a 30K MP and the triple gene block. Indeed, carmoviruses (family *Tombusviridae*) encode two small proteins of 7 and 9 kDa, sometimes referred to as double gene block proteins, which are essential for intra- and inter-cellular movement but are unrelated to either 30K or triple gene block MPs,[193] further indicating that the ability to cross plasmodesmata evolved on several occasions. In yet other plant viruses, such as closteroviruses, the functions of the MP are performed by minor virion components including the aforementioned virus-encoded HSP70 family chaperone,[10,15,204] another case of gene sharing.

In addition to the MPs, numerous plant and insect viruses encode proteins, in some cases, more than one per genome, known as silencing suppressors, that inhibit the powerful RNAi systems of their hosts.[40,46,105] Most of the silencing suppressors are small, structurally diverse proteins. Although many show no significant similarity to any known proteins,[163] some RNA and DNA viruses encode silencing suppressors containing the dsRNA binding domain (dsRBD), a small protein motif of ~70 amino acids with an αββββα fold, whose key property is binding dsRNA.[62] It cannot be ruled out that some of the silencing suppressors evolved de novo from spurious open reading frames (ORFs) in viral genomes.

Several small groups of plant positive-sense RNA viruses in the order *Tymovirales* within *Kitrinoviricota* and family *Potyviridae* within *Pisuviricota* encode an RNA demethylase of the AlkB family that repairs oxidative damage in virus RNAs.[12,247] It remains unclear why some but not other plant viruses require this enzyme for their reproduction. One possibility is that the AlkB homolog is present mostly in viruses infecting trees that might exhibit a high level of oxidative stress.

Connections to the host biology are tractable for other viruses as well, especially in well-studied, model host organisms. Thus, most of bacteriophages encode peptidoglycan digesting enzymes (lysins) or other proteins that cause lysis of the cell wall allowing virus egress.[30,31] The lysins belong to several unrelated families of peptidoglycan-cleaving enzymes, indicative of independent capture by different groups of phages.[1] Remarkably, even leviviruses and microviruses, ssRNA and ssDNA phages with extremely compact genomes, respectively, encode distinct lysis proteins which, in these cases, are small membrane proteins encoded in alternative ORFs that cause lysis by inhibiting different stages of the peptidoglycan biosynthesis pathway.[32] Apparently, these are independent cases of de novo emergence of virus proteins whereby spurious ORFs were fixed in evolution under strong selective pressure for the essential lysis activity. Indeed, leviviruses encode several unrelated lysis proteins that were overprinted over different viral genes.[33]

Viruses infecting biologically distinct groups of bacteria display more specific adaptations. Notable examples are the numerous viruses of cyanobacteria (cyanophages), many of which carry genes for the entire photosystem I, photosystem II, or both.[8,70,224,235] The photosystems of cyanophages supplement those of the host cyanobacteria, maintaining the host metabolism at the level sufficient to support the maximum rate of the virus reproduction. Such complementation of the host metabolism that is either overtasked by the virus infection or shut down in response to it is likely to be the driving force behind the capture of a variety of metabolic enzymes by viruses with large DNA genomes. In particular, the widespread nucleoside and nucleotide kinases discussed above and other enzymes of nucleotide metabolism that are common in viruses, for example, ribonucleotide reductases and dihydrofolate reductases, belong in this functional category.[93,168]

A particularly notable example of recruitment of proteins complementing host functions that merits special discussion are protein components of the translation systems that are encoded by many giant viruses, primarily, in the family *Mimiviridae* within *Nucleocytovirecetes*. The most striking case is tupanvirus that encodes all protein components of the translation machinery except for the ribosomal proteins, including the complete set of aminoacyl-tRNA synthetases (aaRS) and translation factors.[3,217] Although the functions on these proteins have not been studied in the native virus-host systems, the activity of some of the aaRS has been demonstrated *in vitro*, and the conservation of the catalytic amino acids indicates that all the viral aaRS are active.[2] Thus, it appears highly likely that virus proteins complement rather than inhibit the host translation system. The presence of multiple translation system components among the mimivirus proteins fueled speculation on the origin of giant viruses from cellular ancestors by reductive evolution.[38,153,192] However, the distribution of the translation system components among the giant viruses is extremely patchy, and phylogenetic analysis indicates that these proteins have been acquired piecemeal during virus evolution.[259,270] Notably, a variety of bacterial and archaeal members of *Caudoviricetes* encode varying sets of ribosomal proteins,[181] numerous tRNAs, and diverse tRNA metabolism genes, occasionally, including aaRS.[89,168]

Analogously to the silencing repressors in viruses of eukaryotes, viruses of bacteria and archaea encode many proteins that target the prokaryote defense machinery, in particular CRISPR adaptive immune systems. Thanks to the CRISPR boom, the anti-CRISPR proteins (ACRs) have been studied in greater detail than other antidefense proteins of prokaryote viruses.[41,124,158,257] The ACRs show enormous structural and functional diversity.[257] The spread of the ACRs among bacterial and archaeal viruses is extremely nonuniform. Some viruses, for example, certain

rudiviruses in the realm *Adnaviria*, encode multiple ACRs that can account for up to 25% of the genome coding capacity, whereas other, related viruses appear to encode many fewer ACRs or none at all.[14,21,88] There is little sequence and even structure conservation among the ACRs from different viruses except for closely related ones, suggestive of rapid sequence evolution and, possibly, de novo emergence of these proteins, again, analogously to the silencing suppressors of plant viruses. Most of the ACRs are small proteins that inhibit a specific variety of CRISPR systems by binding to different parts of the large CRISPR effector complexes (or multidomain effector proteins) and blocking their activity. Some of these proteins are DNA mimics that form unproductive complexes with the CRISPR effectors.[71,166,225] Most of the ACRs lack any enzymatic activity, but there are notable exceptions, such as the viral ring nuclease that is widely spread among bacterial and archaeal viruses, and cleaves cyclic oligoadenylates, abrogating dormancy or cell death induction by type III CRISPR systems that is triggered by these second signal molecules.[14] Notably, this viral ring nuclease adopts a unique protein fold, so far not detected in any other proteins, making its origin enigmatic. Computational methods for ACR prediction from viral genome sequences that have been developed on the basis of salient characteristics of the ACR proteins and genes, such as small size, close gene spacing, and adjacency to genes encoding transcription repressors regulating their expression, indicate that bacterial and archaeal viruses within *Duplodnaviria* and *Adnaviria* encode numerous, still uncharacterized ACRs.[86] Moreover, diverse proteins encoded by prokaryote viruses, some resembling the ACRs in general properties, including the lack of readily identifiable origin, most likely, inhibit other defense systems of bacteria and archaea.[20]

Antidefense systems probably have been studied in greatest detail in chordopoxviruses, in which about 100 of the ~200 encoded proteins have dedicated functions in antidefense.[223] Multiple chordopoxvirus proteins block different innate immunity pathways, in particular, the interferon-mediated resistance, the ubiquitin-proteasome system, and apoptosis, often acting as dominant negative inhibitors.[26,186,187] For example, vaccinia virus (VACV) protein E3 contains a dsRNA-binding domain and functions as a dominant negative inhibitor of the PKR kinase through the formation of unproductive complexes with dsRNA, preventing activation of PKR.[151,176] An unrelated protein, K3, is a structural mimic of the translation initiation factor eIF2a and inhibits via completely different mechanism, directly binding the kinase.[57] Notably, chordopoxviruses encompass four families of defense proteins, each consisting of several paralogs, two of which include proteins containing repetitive structures, namely, Kelch and TPR repeats, as well as Bcl-2 domains inhibiting different stages of apoptosis and so-called PIE (Poxvirus Immune Evasion) domains.[223] The Bcl-2 proteins are a typical case of recruitment of host proteins as dominant negative inhibitors of the respective defense pathways.[149,243] Phylogenetic analysis shows that these protein families evolved by serial gene duplication during poxvirus evolution, following the initial capture by an ancestral chordopoxvirus.[223] The origins of most of these proteins can be generically traced to animal ancestors, but not at the level of specific genes. Moreover, the PIE domain fold appears to be unique, with no similarity to any available protein structures.[194] Taken together, these observations indicate that most of the virus antidefense proteins are substantially altered upon recruitment by viruses, in some cases, even through core fold rearrangements. However, there are some exceptions to this trend, when an apparently recently acquired protein retains high sequence similarity to an obvious ancestor in the host, for example, serpins, inhibitors of caspases that prevent apoptosis.[26]

The repertoire of poxvirus antidefense proteins fully depends on the host biology. Indeed, fish poxviruses, the deepest branch among chordopoxviruses, lack all the characteristic antidefense proteins present in the rest of the group and instead encode a roughly equivalent number of proteins without detectable similarity in the current database.[73] By implication, these proteins can be suspected to function in antidefense, but no specific indications can be gleaned from the analysis of their sequences.

Dark Matter of Virus Proteomes

Fast evolution that often obscures ancestry is arguably one of the key features of virus proteins. Despite the small genome size of most RNA viruses, even these encode many proteins and domains without detectable homologs outside the respective group of viruses. For example, a substantial part of the nidovirus polyproteins remains completely uncharacterized despite conservation in related viruses.[85,220] In the preceding section, we mention silencing suppressors encoded by diverse plant viruses. Most of these are small proteins without detectable homologs among known proteins, and the only information on these proteins comes from direct experiments on a handful of model viruses. The enormous variety of uncharacterized viruses encode their own “ORFans” (a nickname for uncharacterized, poorly conserved ORFs) that can be tentatively implicated in antidefense and perhaps other aspects of virus-host interactions, but their specific functions are currently unpredictable, and their origins remain obscure.[263] The ssDNA viruses in the realm *Monodnaviria* have tiny genomes with few genes, and the encoded proteins consist of known domains except, again, for some uncharacterized small proteins. A distinct family of viruses with small ssDNA genomes, *Anelloviridae*, common commensals of animals including humans, encode several proteins, none of which has detectable homologs outside this virus family.[108,254] Using highly sensitive methods for protein structure prediction, it became possible to determine that the CP of anelloviruses actually is a highly derived SJR protein, but the rest remain dark matter. The viruses with the smallest known genomes, members of the realm *Ribozyviria* that includes human hepatitis delta virus (HDV) and its relatives from other animals,[205] are thought to originate from viroidlike, noncoding infectious agents and encode a single protein that forms the virus nucleocapsid.[128,152] No homologs of this protein have been detected so far. Thus, the entire proteome of this virus realm is dark matter.

In viruses with larger dsDNA genomes, the fraction of protein dark matter differs widely. In the well-characterized poxviruses and herpesviruses, the domain architectures as well as biochemical and biological functions of most proteins are known, and the origins of many from cellular ancestors are apparent, even if not down to a specific host gene, due to the high divergence of most virus proteins. However, in other viruses with large genomes, in particular, the giant pandoraviruses,[154] most of the proteins represent dark matter. An even higher fraction of such ORFans, up to 90% of the (predicted) proteins, has been reported for large dsDNA viruses that currently could not be classified into any of the two major realms, such a nimaviruses[253] and yaravirus,[23] in which all proteins except for a few VHP remain complete unknowns.

The dark matter of virus proteomes can be classified into two categories that can be colloquially dubbed “knowable unknowns” and “unknowable unknowns,” respectively. The first category consists of highly derived descendants of cellular proteins whereas the second one includes proteins that might have originated in viruses *de novo*, from noncoding sequences. For the “knowable unknowns,” light can be shed on their origins through the application of sensitive methods for detection of sequence and structure similarity. A notable case in point is the large group of the so-called crAss-like phages that includes some of the most abundant phages infecting bacteria in the human gut microbiome.[54] When the first crAssphage was discovered, no homologs were detected for more than 80% of its predicted proteins, and even the expected VHP, such as the HK97 fold CP, were not identified.[52] However, subsequent analysis that combined the discovery of numerous related phages and search of their protein sequences using sensitive methods established the identity and likely origins of the majority of these unknowns, leaving only about one-third of the proteins within the dark matter.[267,269] With the decisive progress made in the field of protein structure prediction, with AlphaFold2[107] and RoseTTAFold,[17] it can be expected that numerous knowable unknowns among virus proteins leave the dark matter realm

soon.

Many other virus proteins, however, such as the silencing suppressors in plant viruses and ACRs in bacterial and archaeal viruses, seem to be unknowable unknowns. Typically, these are small proteins that are poorly conserved even among closely related viruses and no homologs detected even using the most sensitive available methods. These proteins can be suspected to have originated de novo although demonstrating this origin definitively is difficult except for proteins that evolve by overprinting, that is, as an alternative reading frame within other genes, for which the *de novo* scenario appears a certainty.[201,202] The lysis proteins of leviviruses are a typical case, and analogous proteins in alternative frames are also encoded by other RNA viruses and some ssDNA viruses.

Exaptation and Recruitment of Host Proteins as Major Routes of Virus Protein Evolution

The massive comparative analyses of virus proteins performed in the last few years provide for some generalizations on the routes of virus proteome evolution. With the apparent exception of the several VHPs that play key roles in virus genome replication and are likely to be direct descendants of enzymes that catalyzed the replication of primordial replicators, the rest of virus evolution appears to be largely a history of exaptation (Fig. 12.9). Exaptation was defined by Gould and Vrba as recruitment of biological structures for roles distinct from their original functions shaped by adaptation.[81] Although originally formulated in the context of organismal biology, the concept of exaptation is readily expandable and is a powerful explanatory framework for many aspects of molecular evolution. In virus evolution, exaptation is traceable back to the earliest stage, the recruitment of cellular carbohydrate-binding proteins as viral CPs, in particular, the SJR domains that apparently were recruited on several independent occasions.[138] Notably, despite the original reconstructions of the evolution of the DJR CP via SJR duplication in a virus genome, a more detailed investigation revealed an independent exaptation, again of a carbohydrate-active enzyme, a glycoside hydrolase.[144] Characteristically, the giant pandoraviruses that lost the ancestral DJR CP of *Nucleocytovirecetes* (and with it, the icosahedral capsid itself) also recruited an inactivated glycoside hydrolase of a different family as their major virion protein.[145] In small viruses, the principal adaptation of the CPs for their role in the virions is the acquisition of positively charged residues that interact with the virus genomic RNA or DNA. Typically, these positive charges reside in terminal extensions of the CPs such that exaptation does not substantially affect the core protein fold.

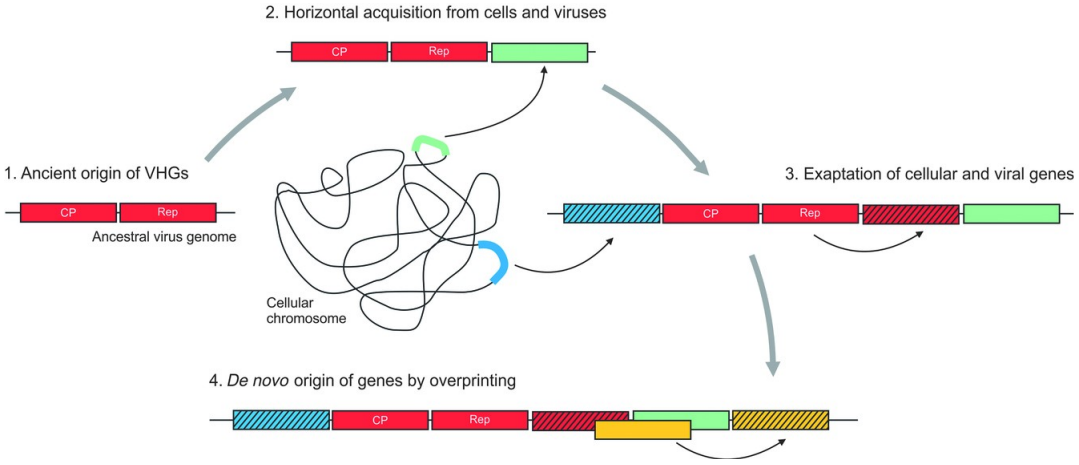

FIGURE 12.9 **Routes of evolution of viral proteins**.

Several other cases of emergence of major virion protein via exaptation of host proteins have been documented.[138] One notable example is the matrix protein of retroviruses, which is a derivative of the DNA-binding helix-turn-helix domain of transposon integrases. Another case in point are the matrix proteins of the viruses in the order *Mononegavirales* (phylum *Negarnaviricota*) that appear to have been derived from cyclophilins, molecular chaperones with peptidyl-prolyl-isomerase activity. When discussing the evolution of CPs above, we already mentioned the CP of alphaviruses, which is a derived chymotrypsinlike protease. In this case, the route of exaptation was apparently complex, with the protease first acquired by members of *Flaviviridae* and employed for polyprotein processing, followed by the gene transfer to alphaviruses, in which exaptation for the CP function occurred. The last two examples illustrate an important trend of exaptive evolution of virus proteins, namely, recruitment of enzymes for structural roles accompanied by loss of the enzymatic activity.

The numerous cases of recruitment of cellular proteins by viruses span a wide range of protein adaptation and evolutionary modification. Some of the recruited proteins effectively retain the same ancestral functions, for instance, enzymes involved in virus DNA replication or nucleotide metabolism. In such directly recruited viral enzymes, not only the protein fold but also the catalytic amino acids are conserved, and accordingly, the similarity to the cellular ancestors remains pronounced. Then, there are many exaptations that involve a limited change to the respective protein, notably, in the cases when a protein recruited by viruses functions as a mimic of a host protein involved in a particular antivirus defense pathway and hence a dominant negative inhibitor of that pathway. Soluble cytokine receptors and DEATH domain functioning as apoptosis inhibitors, both encoded by poxviruses, are good examples of such evolutionary strategy.[9,265] These proteins typically are represented in relatively narrow groups of viruses. The cellular ancestry of such proteins is readily discernible, typically, via straightforward sequence comparison. The CPs discussed above are farther removed from their cellular ancestors on the spectrum of virus exaptations and are only detectable by advanced sequence analysis approaches or by structure comparison. In these cases, there is a clear mechanistic similarity between the original function of a protein and that in viruses, even though the biological roles are quite different. Finally, some virus proteins evolve via exaptation of cellular proteins for functions that are not obviously related to the original ones. The discovery of ancestry is such cases comes as a surprise and often requires the most sensitive and/or structural comparisons. A relevant example is the poxvirus structural protein F12 that turned out to be a derived, inactivated DNA polymerase.[268] Similarly, in herpesviruses, protein UL8 is an inactivated family B DNA polymerase, which was recruited to function as a nonenzymatic, yet essential component of the helicase-primase complex, implicated in multiple protein-protein interactions.[110] It appears likely that the extent of such radical exaptation of host proteins by viruses is substantially underappreciated because of the major changes of the involved protein sequences and even structures. Many more such cases are likely to be discovered through comprehensive prediction of virus protein structures using the new, powerful methods.

Concluding Remarks: Organization of the Virus Pan-proteome and Challenges in its Analysis

The virus pan-proteome, that is, the entirety of proteins encoded by viruses, is the largest protein pool on earth, apparently spanning a much greater region of the sequence space than the pan-proteome of all cellular life forms combined. This is the case because viruses with large genomes encode numerous proteins unique to narrow groups of viruses. Our knowledge of the diversity of viromes appears insufficient for a robust quantitative estimate of the size of the virus pan-proteome, but beyond doubt, there are in the least many millions of groups of related viruses and hence billions of virus proteins. A key common feature of virus proteins is their fast, albeit widely varying, evolutionary change. At coarse grain and with some degree of arbitrariness, the virus pan-proteome can be classified into four classes of proteins that differ in their evolutionary conservation and spread across the virosphere (Fig. 12.10):

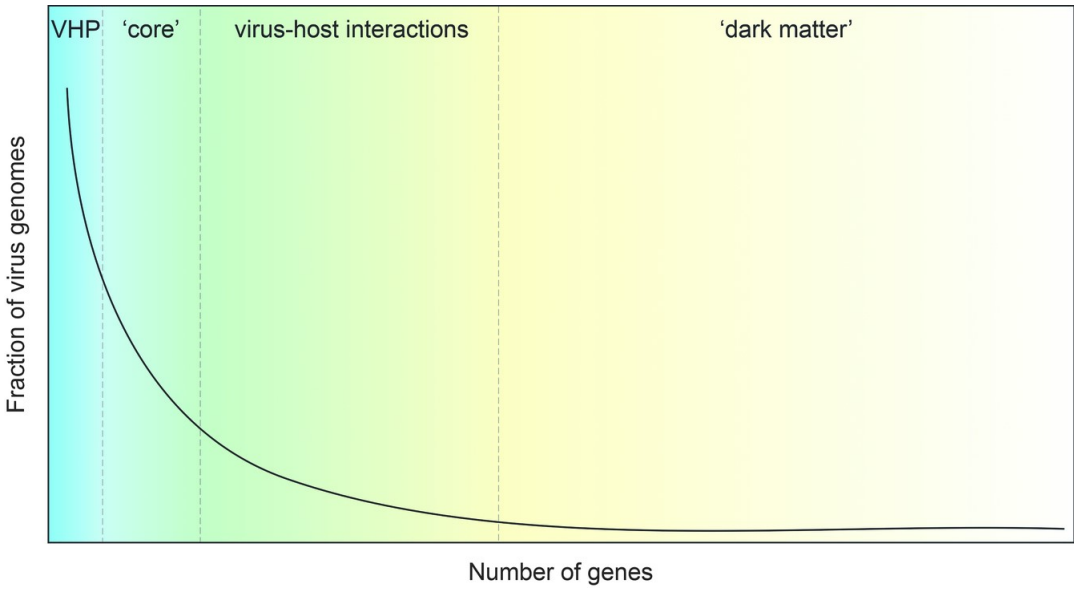

FIGURE 12.10 **Frequency of the four functional gene categories in viral genomes**. The curve is approximate, based on the results of comparative analysis of virus genomes, but not reflecting exact numbers.

i. the VHPs that are broadly conserved across different realms of viruses.

ii. proteins involved in core virus functions, such as replication, expression, and virion formation, but typically represented within a single virus realm or kingdom.

iii. proteins involved in virus-host interactions, primarily, counteracting host defenses, typically, conserved only in narrow groups of viruses and evolving at a high rate.

iv. dark matter, that is, proteins without detectable homologs or known functions, almost in all cases shared only by closely related viruses. The boundary between the latter two classes of virus proteins is, obviously, porous, so that as the provenance and functionality of the dark matter proteins are clarified, they migrate into the virus-host interaction category. Indeed, most of the current unknowns can be expected to be involved in various virus-host interactions.

Apart from the few VHPs that seem to originate from the primordial protein pool, the bulk of the virus pan-proteome is derived from cellular proteins, via direct recruitment of proteins with activities relevant for virus reproduction as well as extensive exaptation. The degree of change to the sequences and structures of proteins acquired by viruses from hosts reflects the extent of functional change. Genes encoding recruited proteins that retain their activities may experience a period of accelerated evolution once integrated into virus genomes, but the magnitude of such acceleration is constrained by the requirement for preservation of active sites, in addition to the protein fold. In contrast, exaptation events cover a broad range of sequence and structure change, from high level of sequence conservation despite a functional switch to extreme divergence, including structural rearrangement that can make the ancestry of virus proteins hard if possible at all to trace. Comprehensive experimental and computational analyses of virus protein structures have the potential to reveal many more cases of radical protein change accompanying exaptation.

In addition to the recruitment and exaptation of host proteins, virus genomes might be a hotbed of *de novo* protein emergence. This route of protein origin is obvious in cases of overprinting, but hard to convincingly demonstrate on other occasions. Nevertheless, *de novo* origin of numerous small proteins encoded by diverse viruses, for example ACRs, appears plausible and could be an important source of new protein folds.

Virus proteomes, clearly, are not random collections of proteins. Rather, the recruitment of cellular proteins for roles in viral reproduction follows a logic dictated by functionalities that are relevant for viruses. Under this logic, different groups of viruses convergently capture distinct, often unrelated proteins providing similar functionalities, for example, helicases and proteases of several different families. Further, viruses acquire and possibly evolve de novo diverse proteins that target the defense systems that are most prominent in their respective hosts, such as CRISPR in bacteria and archaea, RNAi in plants, and apoptosis in animals. An important part of the logic of virus evolution is the "guns for hire" trend whereby viruses capture components of the host defense systems and repurpose them for antidefense, for example, as dominant negative inhibitors.[131]

Concluding the discussion of the major trends in the evolution of virus proteins and proteomes, it seems prudent to mention some serious challenges confronted by the analysis of deep connections among viral proteins. The strongest challenge, arguably, is the fast pace of change of viral protein sequences and even structures, because of which many relationships can remain unnoticed. Comprehensive structural

characterization of viral proteomes is key. With the advent of the latest generation of structure modeling methods, this has become a realistic prospect, but the work itself and careful analysis and interpretation of the results still lie in the future. Fast evolution of viral proteins, especially, perhaps, at times of evolutionary transitions that include emergence of new groups of viruses also seriously compromises the robustness of phylogenetic analyses. Methodological improvements are badly needed and may come from refined methods for phylogenetic tree construction based on direct protein structure comparisons. Further characterization of the diversity of the virus proteomes combined with in-depth structural and functional studies of virus proteins using improved methods can be expected to clarify the architecture of the virus pan-proteome and the logic of virus evolution.

REFERENCES

1. Abdelrahman F, Easwaran M, Daramola OI, et al. Phage-encoded endolysins. Antibiotics (Basel). 2021;10:124.

2. Abergel C, Rudinger-Thirion J, Giege R, et al. Virus-encoded aminoacyl-tRNA synthetases: structural and functional characterization of mimivirus TyrRS and MetRS. J Virol. 2007;81:12406-12417.

3. Abrahao J, Silva L, Silva LS, et al. Tailed giant Tupanvirus possesses the most complete translational apparatus of the known virosphere. Nat Commun. 2018;9:749.

4. Abrescia NG, Bamford DH, Grimes JM, et al. Structure unifies the viral universe. Annu Rev Biochem. 2012;81:795-822.

5. Agol VI, Gmyl AP. Viral security proteins: counteracting host defences. Nat Rev Microbiol. 2010;8:867-878.

6. Ahola T, Karlin DG. Sequence analysis reveals a conserved extension in the capping enzyme of the alphavirus supergroup, and a homologous domain in nodaviruses. Biol Direct. 2015;10:16.

7. Ahola T, McInerney G, Merits A. Alphavirus RNA replication in vertebrate cells. Adv Virus Res. 2021;111:111-156.

8. Alperovitch-Lavy A, Sharon I, Rohwer F, et al. Reconstructing a puzzle: existence of cyanophages containing both photosystem-I and photosystem-II gene suites inferred from oceanic metagenomic datasets. Environ Microbiol. 2011;13:24-32.

9. Alvarez-de Miranda FJ, Alonso-Sanchez I, Alcami A, et al. TNF decoy receptors encoded by poxviruses. Pathogens. 2021;10:1065.

10. Alzhanova DV, Napuli AJ, Creamer R, et al. Cell-to-cell movement and assembly of a plant closterovirus: roles for the capsid proteins and Hsp70 homolog. EMBO J. 2001;20:6997-7007.

11. Ansarah-Sobrinho C, Moss B. Role of the I7 protein in proteolytic processing of vaccinia virus membrane and core components. J Virol. 2004;78:6335-6343.

12. Aravind L, Koonin EV. The DNA-repair protein AlkB, EGL-9, and leprecan define new families of 2-oxoglutarate- and iron-dependent dioxygenases. Genome Biol. 2001;2:RESEARCH0007.

13. Aravind L, Zhang D, de Souza RF, et al. The natural history of ADP-ribosyltransferases and the ADP-ribosylation system. Curr Top Microbiol Immunol. 2015;384:3-32.

14. Athukoralage JS, McMahon SA, Zhang C, et al. An anti-CRISPR viral ring nuclease subverts type III CRISPR immunity. Nature. 2020;577:572-575.

15. Avisar D, Prokhnevsky AI, Dolja VV. Class VIII myosins are required for plasmodesmatal localization of a closterovirus Hsp70 homolog. J Virol. 2008;82:2836-2843.

16. Aylward FO, Moniruzzaman M, Ha AD, et al. A phylogenomic framework for charting the diversity and evolution of giant viruses. PLoS Biol. 2021;19:e3001430.

17. Baek M, DiMaio F, Anishchenko I, et al. Accurate prediction of protein structures and interactions using a three-track neural network. Science. 2021;373:871-876.

18. Bailey-Elkin BA, Knaap RCM, Kikkert M, et al. Structure and function of viral deubiquitinating enzymes. J Mol Biol. 2017;429:3441-3470.

19. Baltimore D. Expression of animal virus genomes. Bacteriol Rev. 1971;35:235-241.

20. Bernheim A, Sorek R. The pan-immune system of bacteria: antiviral defence as a community resource. Nat Rev Microbiol. 2020;18:113-119.

21. Bhoobalan-Chitty Y, Johansen TB, Di Cianni N, et al. Inhibition of type III CRISPR-Cas immunity by an archaeal virus-encoded anti-CRISPR protein. Cell. 2019;179:448-458 e411.

22. Black LW, Rao VB. Structure, assembly, and DNA packaging of the bacteriophage T4 head. Adv Virus Res. 2012;82:119-153.

23. Boratto PVM, Oliveira GP, Machado TB, et al. Yaravirus: a novel 80-nm virus infecting *Acanthamoeba castellanii*. Proc Natl Acad Sci U S A. 2020;117:16579-16586.

24. Born D, Reuter L, Mersdorf U, et al. Capsid protein structure, self-assembly, and processing reveal morphogenesis of the marine virophage mavirus. Proc Natl Acad Sci U S A. 2018;115:7332-7337.

25. Bouvet M, Debarnot C, Imbert I, et al. In vitro reconstitution of SARS-coronavirus mRNA cap methylation. PLoS Pathog. 2010;6:e1000863.

26. Bratke KA, McLysaght A, Rothenburg S. A survey of host range genes in poxvirus genomes. Infect Genet Evol. 2013;14:406-425.

27. Broyles SS, Moss B. Homology between RNA polymerases of poxviruses, prokaryotes, and eukaryotes: nucleotide sequence and

transcriptional analysis of vaccinia virus genes encoding 147-kDa and 22-kDa subunits. Proc Natl Acad Sci U S A. 1986;83:3141-3145.

28. Burroughs AM, Iyer LM, Aravind L. Comparative genomics and evolutionary trajectories of viral ATP dependent DNA-packaging systems. Genome Dyn. 2007;3:48-65.

29. Byrd CM, Bolken TC, Hruby DE. The vaccinia virus I7L gene product is the core protein proteinase. J Virol. 2002;76:8973-8976.

30. Cahill J, Young R. Phage lysis: multiple genes for multiple barriers. Adv Virus Res. 2019;103:33-70.

31. Chamakura K, Young R. Phage single-gene lysis: Finding the weak spot in the bacterial cell wall. J Biol Chem. 2019;294:3350-3358.

32. Chamakura KR, Young R. Single-gene lysis in the metagenomic era. Curr Opin Microbiol. 2020;56:109-117.

33. Chamakura KR, Tran JS, O'Leary C, et al. Rapid de novo evolution of lysis genes in single-stranded RNA phages. Nat Commun. 2020;11:6009.

34. Chandler M, de la Cruz F, Dyda F, et al. Breaking and joining single-stranded DNA: the HUH endonuclease superfamily. Nat Rev Microbiol. 2013;11:525-538.

35. Cheng H, Shen N, Pei J, et al. Double-stranded DNA bacteriophage prohead protease is homologous to herpesvirus protease. Protein Sci. 2004;13:2260-2269.

36. Claverie JM. Fundamental difficulties prevent the reconstruction of the deep phylogeny of viruses. Viruses. 2020;12:1130.

37. Clery A, Blatter M, Allain FH. RNA recognition motifs: boring? Not quite. Curr Opin Struct Biol. 2008;18:290-298.

38. Colson P, de Lamballerie X, Fournous G, et al. Reclassification of giant viruses composing a fourth domain of life in the new order Megavirales. Intervirology. 2012;55:321-332.

39. Croft SN, Walker EJ, Ghildyal R. Picornaviruses and apoptosis: subversion of cell death mBio. 2017;8:e01009-17.

40. Csorba T, Kontra L, Burgyan J. viral silencing suppressors: Tools forged to fine-tune host-pathogen coexistence. Virology. 2015;479-480:85-103.

41. Davidson AR, Lu WT, Stanley SY, et al. Anti-CRISPRs: protein inhibitors of CRISPR-Cas systems. Annu Rev Biochem. 2020;89:309-332.

42. De Colibus L, Roine E, Walter TS, et al. Assembly of complex viruses exemplified by a halophilic euryarchaeal virus. Nat Commun. 2019;10:1456.

43. de Martín Garrido N, Orekhova M, Lai Wan Loong YTE, et al. Structure of the bacteriophage PhiKZ non-virion RNA polymerase. Nucleic Acids Res. 2021;49:7732-7739.

44. Deaton MK, Spear A, Faaberg KS, et al. The vOTU domain of highly-pathogenic porcine reproductive and respiratory syndrome virus displays a differential substrate preference. Virology. 2014;454-455:247-253.

45. Decroly E, Ferron F, Lescar J, et al. Conventional and unconventional mechanisms for capping viral mRNA. Nat Rev Microbiol. 2011;10:51-65.

46. Deng Z, Ma L, Zhang P, et al. Small RNAs participate in plant-virus interaction and their application in plant viral defense. Int J Mol Sci. 2022;23:696.

47. Dolja VV, Kreuze JF, Valkonen JP. Comparative and functional genomics of closteroviruses. Virus Res. 2006;117:38-51.

48. Drobysheva AV, Panafidina SA, Kolesnik MV, et al. Structure and function of virion RNA polymerase of a crAss-like phage. Nature. 2021;589:306-309.

49. Duda RL, Teschke CM. The amazing HK97 fold: versatile results of modest differences. Curr Opin Virol. 2019;36:9-16.

50. Duda RL, Oh B, Hendrix RW. Functional domains of the HK97 capsid maturation protease and the mechanisms of protein encapsidation. J Mol Biol. 2013;425:2765-2781.

51. Duffy S, Shackelton LA, Holmes EC. Rates of evolutionary change in viruses: patterns and determinants. Nat Rev Genet. 2008;9:267-276.

52. Dutilh BE, Cassman N, McNair K, et al. A highly abundant bacteriophage discovered in the unknown sequences of human faecal metagenomes. Nat Commun. 2014;5:4498.

53. Dzimianski JV, Beldon BS, Daczkowski CM, et al. Probing the impact of nairovirus genomic diversity on viral ovarian tumor domain protease (vOTU) structure and deubiquitinase activity. PLoS Pathog. 2019;15:e1007515.

54. Edwards RA, Vega AA, Norman HM, et al. Global phylogeography and ancient evolution of the widespread human gut virus crAssphage. Nat Microbiol. 2019;4:1727-1736.

55. Egloff MP, Malet H, Putics A, et al. Structural and functional basis for ADP-ribose and poly(ADP-ribose) binding by viral macro domains. J Virol. 2006;80:8493-8502.

56. El Omari K, Meier C, Kainov D, et al. Tracking in atomic detail the functional specializations in viral RecA helicases that occur during evolution. Nucleic Acids Res. 2013;41:9396-9410.

57. Elde NC, Child SJ, Geballe AP, et al. Protein kinase R reveals an evolutionary model for defeating viral mimicry. Nature. 2009;457:485-489.

58. Enemark EJ, Chen G, Vaughn DE, et al. Crystal structure of the DNA binding domain of the replication initiation protein E1 from papillomavirus. Mol Cell. 2000;6:149-158.

59. Erives AJ. Phylogenetic analysis of the core histone doublet and DNA topo II genes of Marseilleviridae: evidence of proto-eukaryotic provenance. Epigenetics Chromatin. 2017;10:55.

60. Fang Q, Zhu D, Agarkova I, et al. Near-atomic structure of a giant virus. Nat Commun. 2019;10:388.

61. Ferron F, Sama B, Decroly E, et al. The enzymes for genome size increase and maintenance of large (+)RNA viruses. Trends Biochem Sci. 2021;46:866-877.

62. Fierro-Monti I, Mathews MB. Proteins binding to duplexed RNA: one motif, multiple functions. Trends Biochem Sci. 2000;25:241-246.

63. Filee J, Forterre P. Viral proteins functioning in organelles: a cryptic origin? Trends Microbiol. 2005;13:510-513.

64. Fokine A, Rossmann MG. Common evolutionary origin of procapsid proteases, phage tail tubes, and tubes of bacterial type VI secretion systems. Structure. 2016;24:1928-1935.

65. Forterre P. The two ages of the RNA world, and the transition to the DNA world: a story of viruses and cells. Biochimie. 2005;87:793-803.

66. Forterre P. Three RNA cells for ribosomal lineages and three DNA viruses to replicate their genomes: a hypothesis for the origin of cellular domain. Proc Natl Acad Sci U S A. 2006;103:3669-3674.

67. Forterre P, Prangishvili D. The great billion-year war between ribosome- and capsid-encoding organisms (cells and viruses) as the major source of evolutionary novelties. Ann N Y Acad Sci. 2009;1178:65-77.

68. Forterre P, Krupovic M, Prangishvili D. Cellular domains and viral lineages. Trends Microbiol. 2014;22:554-558.

69. Fournier GP, Andam CP, Gogarten JP. Ancient horizontal gene transfer and the last common ancestors. BMC Evol Biol. 2015;15:70.

70. Fridman S, Flores-Uribe J, Larom S, et al. A myovirus encoding both photosystem I and II proteins enhances cyclic electron flow in infected Prochlorococcus cells. Nat Microbiol. 2017;2:1350-1357.

71. Gabel C, Li Z, Zhang H, et al. Structural basis for inhibition of the type I-F CRISPR-Cas surveillance complex by AcrIF4, AcrIF7 and AcrIF14. Nucleic Acids Res. 2021;49:584-594.

72. Gardberg A, Shuvalova L, Monnerjahn C, et al. Structural basis for the dual thymidine and thymidylate kinase activity of herpes thymidine kinases. Structure. 2003;11:1265-1277.

73. Gjessing MC, Yutin N, Tengs T, et al. Salmon gill poxvirus, the deepest representative of the chordopoxvirinae. J Virol. 2015;89:9348-9367.

74. Goodfellow I. The genome-linked protein V—Pg of vertebrate viruses—a multifaceted protein. Curr Opin Virol. 2011;1:355-362.

75. Gorbalenya AE, Donchenko AP, Blinov VM, et al. Cysteine proteases of positive strand RNA viruses and chymotrypsin-like serine proteases. A distinct protein superfamily with a common structural fold. FEBS Lett. 1989;243:103-114.

76. Gorbalenya AE, Enjuanes L, Ziebuhr J, et al. Nidovirales: evolving the largest RNA virus genome. Virus Res. 2006;117:17-37.

77. Gorbalenya AE, Koonin EV, Lai MM. Putative papain-related thiol proteases of positive-strand RNA viruses. Identification of rubi- and aphthovirus proteases and delineation of a novel conserved domain associated with proteases of rubi-, alpha- and coronaviruses. FEBS Lett. 1991;288:201-205.

78. Gorbalenya AE, Koonin EV, Wolf YI. A new superfamily of putative NTP-binding domains encoded by genomes of small DNA and RNA viruses. FEBS Lett. 1990;262:145-148.

79. Gornik SG, Ford KL, Mulhern TD, et al. Loss of nucleosomal DNA condensation coincides with appearance of a novel nuclear protein in dinoflagellates. Curr Biol. 2012;22:2303-2312.

80. Gould SJ. The exaptive excellence of spandrels as a term and prototype. Proc Natl Acad Sci U S A. 1997;94:10750-10755.

81. Gould SJ, Vrba E. Exaptation—a missing term in the science of form. Paleobiology. 1982;8:4-15.

82. Grininger M, Zeth K, Oesterhelt D. Dodecins: a family of lumichrome binding proteins. J Mol Biol. 2006;357:842-857.

83. Guarino LA, Xu B, Jin J, et al. A virus-encoded RNA polymerase purified from baculovirus-infected cells. J Virol. 1998;72:7985-7991.

84. Guglielmini J, Woo AC, Krupovic M, et al. Diversification of giant and large eukaryotic dsDNA viruses predated the origin of modern eukaryotes. Proc Natl Acad Sci U S A. 2019;116:19585-19592.

85. Gulyaeva AA, Gorbalenya AE. A nidovirus perspective on SARS-CoV-2. Biochem Biophys Res Commun. 2021;538:24-34.

86. Gussow AB, Park AE, Borges AL, et al. Machine-learning approach expands the repertoire of anti-CRISPR protein families. Nat Commun. 2020;11:3784.

87. Harada R, Inagaki Y. Phage origin of mitochondrion-localized family a DNA polymerases in kinetoplastids and diplonemids. Genome Biol Evol. 2021;13.

88. He F, Bhoobalan-Chitty Y, Van LB, et al. Anti-CRISPR proteins encoded by archaeal lytic viruses inhibit subtype I-D immunity. Nat Microbiol. 2018;3:461-469.

89. Hendrix RW. Jumbo bacteriophages. Curr Top Microbiol Immunol. 2009;328:229-240.

90. Higgs PG, Lehman N. The RNA world: molecular cooperation at the origins of life. Nat Rev Genet. 2015;16:7-17.

91. Hillen HS, Bartuli J, Grimm C, et al. Structural basis of poxvirus transcription: transcribing and capping vaccinia complexes. Cell. 2019;179:1525-1536 e1512.

92. Holm L. DALI and the persistence of protein shape. Protein Sci. 2020;29:128-140.

93. Ignacio-Espinoza JC, Sullivan MB. Phylogenomics of T4 cyanophages: lateral gene transfer in the 'core' and origins of host genes. Environ Microbiol. 2012;14:2113-2126.

94. Ilyina TV, Koonin EV. Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria. Nucleic Acids Res. 1992;20:3279-3285.

95. International Committee on Taxonomy of Viruses Executive Committee. The new scope of virus taxonomy: partitioning the virosphere into 15 hierarchical ranks. Nat Microbiol. 2020;5:668-674.

96. Iranzo J, Krupovic M, Koonin EV. The double-stranded DNA virosphere as a modular hierarchical network of gene sharing. MBio. 2016;7:e00978-16.

97. Irwin NAT, Martin BJE, Young BP, et al. Viral proteins as a potential driver of histone depletion in dinoflagellates. Nat Commun. 2018;9:1535.

98. Irwin NAT, Pittis AA, Richards TA, et al. Systematic evaluation of horizontal gene transfer between eukaryotes and viruses. Nat Microbiol. 2022;7:327-336.

99. Iyer LM, Abhiman S, Aravind L. A new family of polymerases related to superfamily A DNA polymerases and T7-like DNA-dependent RNA polymerases. Biol Direct. 2008;3:39.

100. Iyer LM, Koonin EV, Leipe DD, et al. Origin and evolution of the archaeo-eukaryotic primase superfamily and related palm-domain proteins: structural insights and new members. Nucleic Acids Res. 2005;33:3875-3896.

101. Iyer LM, Makarova KS, Koonin EV, et al. Comparative genomics of the FtsK-HerA superfamily of pumping ATPases: implications for the origins of chromosome segregation, cell division and viral capsid packaging. Nucleic Acids Res. 2004;32:5260-5279.

102. Jackson T, Belsham GJ. Picornaviruses: a view from 3A. Viruses. 2021;13:456.

103. Jeruzalmi D, Steitz TA. Structure of T7 RNA polymerase complexed to the transcriptional inhibitor T7 lysozyme. EMBO J. 1998;17:4101-4113.

104. Jiang J, Laliberte JF. The genome-linked protein VPg of plant viruses—a protein with many partners. Curr Opin Virol. 2011;1:347-354.

105. Jin Y, Zhao JH, Guo HS. Recent advances in understanding plant antiviral RNAi and viral suppressors of RNAi. Curr Opin Virol. 2021;46:65-72.

106. Jones R, Bragagnolo G, Arranz R, et al. Capping pores of alphavirus nsP1 gate membranous viral replication factories. Nature. 2021;589:615-619.

107. Jumper J, Evans R, Pritzel A, et al. Highly accurate protein structure prediction with AlphaFold. Nature. 2021;596:583-589.

108. Kaczorowska J, van der Hoek L. Human anelloviruses: diverse, omnipresent and commensal members of the virome. FEMS Microbiol Rev. 2020;44:305-313.

109. Kainov DE, Mancini EJ, Telenius J, et al. Structural basis of mechanochemical coupling in a hexameric molecular motor. J Biol Chem. 2008;283:3607-3617.

110. Kazlauskas D, Venclovas C. Herpesviral helicase-primase subunit UL8 is inactivated B-family polymerase. Bioinformatics. 2014;30:2093-2097.

111. Kazlauskas D, Krupovic M, Guglielmini J, et al. Diversity and evolution of B-family DNA polymerases. Nucleic Acids Res. 2020;48:10142-10156.

112. Kazlauskas D, Krupovic M, Venclovas C. The logic of DNA replication in double-stranded DNA viruses: insights from global analysis of viral genomes. Nucleic Acids Res. 2016;44:4551-4564.

113. Kazlauskas D, Varsani A, Koonin EV, et al. Multiple origins of prokaryotic and eukaryotic single-stranded DNA viruses from bacterial and archaeal plasmids. Nat Commun. 2019;10:3425.

114. Kim JG, Kim SJ, Cvirkaite-Krupovic V, et al. Spindle-shaped viruses infect marine ammonia-oxidizing thaumarchaea. Proc Natl Acad Sci U S A. 2019;116:15645-15650.

115. Kim JL, Morgenstern KA, Lin C, et al. Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. Cell. 1996;87:343-355.

116. King JA, Dubielzig R, Grimm D, et al. DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. Embo J. 2001;20:3282-3291.

117. Kondratova AA, Neznanov N, Kondratov RV, et al. Poliovirus protein 3A binds and inactivates LIS1, causing block of membrane protein trafficking and deregulation of cell division. Cell Cycle. 2005;4:1403-1410.

118. Koonin EV. Comparative genomics, minimal gene-sets and the last universal common ancestor. Nat Rev Microbiol. 2003;1:127-136.

119. Koonin EV. Temporal order of evolution of DNA replication systems inferred by comparison of cellular and viral DNA polymerases. Biol Direct. 2006;1:39.

120. Koonin EV. Evolution of genome architecture. Int J Biochem Cell Biol. 2009;41:298-306.

121. Koonin EV, Dolja VV. Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. Crit Rev Biochem Mol Biol. 1993;28:375-430.

122. Koonin EV, Ilyina TV. Computer-assisted dissection of rolling circle DNA replication. Biosystems. 1993;30:241-268.

123. Koonin EV, Krupovic M. The depths of virus exaptation. Curr Opin Virol. 2018;31:1-8.

124. Koonin EV, Makarova KS. Anti-CRISPRs on the march. Science. 2018;362:156-157.

125. Koonin EV, Moss B. Viruses know more than one way to don a cap. Proc Natl Acad Sci U S A. 2010;107:3283-3284.

126. Koonin EV, Yutin N. Evolution of the large nucleocytoplasmic DNA viruses of eukaryotes and convergent origins of viral gigantism. Adv Virus Res. 2019;103:167-202.

127. Koonin EV, Dolja VV, Krupovic M, et al. Global organization and proposed megataxonomy of the virus world. Microbiol Mol Biol Rev. 2020;84:e00061-19.

128. Koonin EV, Dolja VV, Krupovic M, et al. Viruses defined by the position of the virosphere within the replicator space. Microbiol Mol Biol Rev. 2021;85:e0019320.

129. Koonin EV, Krupovic M, Agol VI. The Baltimore Classification of viruses 50 years later: how does it stand in the light of virus evolution? Microbiol Mol Biol Rev. 2021;85:e0005321.

130. Koonin EV, Krupovic M, Ishino S, et al. The replication machinery of LUCA: common origin of DNA replication and transcription. BMC Biol. 2020;18:61.

131. Koonin EV, Makarova KS, Wolf YI, et al. Evolutionary entanglement of mobile genetic elements and host defence systems: guns for hire. Nat Rev Genet. 2020;21:119-131.

132. Koonin EV, Senkevich TG, Dolja VV. The ancient Virus World and evolution of cells. Biol Direct. 2006;1:29.

133. Koonin EV, Wolf YI, Nagasaki K, et al. The Big Bang of picorna-like virus evolution antedates the radiation of eukaryotic supergroups. Nat Rev Microbiol. 2008;6:925-939.

134. Krupovic M. Networks of evolutionary interactions underlying the polyphyletic origin of ssDNA viruses. Curr Opin Virol. 2013;3:578-586.

135. Krupovic M, Bamford DH. Virus evolution: how far does the double beta-barrel viral lineage extend? Nat Rev Microbiol. 2008;6:941-948.

136. Krupovic M, Bamford DH. Order to the viral universe. J Virol. 2010;84:12476-12479.

137. Krupovic M, Koonin EV. Polintons: a hotbed of eukaryotic virus, transposon and plasmid evolution. Nat Rev Microbiol. 2015;13:105-115.

138. Krupovic M, Koonin EV. Multiple origins of viral capsid proteins from cellular ancestors. Proc Natl Acad Sci U S A. 2017;114:E2401-E2410.

139. Krupovic M, Koonin EV. Homologous capsid proteins testify to the common ancestry of retroviruses, caulimoviruses, pseudoviruses, and metaviruses. J Virol. 2017;91:e00210-17.

140. Krupovic M, Béguin P, Koonin EV. Casposons: mobile genetic elements that gave rise to the CRISPR-Cas adaptation machinery. Curr Opin Microbiol. 2017;38:36-43.

141. Krupovic M, Dolja VV, Koonin EV. Origin of viruses: primordial replicators recruiting capsids from hosts. Nat Rev Microbiol. 2019;17:449-458.

142. Krupovic M, Dolja VV, Koonin EV. The LUCA and its complex virome. Nat Rev Microbiol. 2020;18:661-670.

143. Krupovic M, Kuhn JH, Wang F, et al. *Adnaviria*: a new realm for archaeal filamentous viruses with linear A-form double-stranded DNA genomes. J Virol. 2021;95:e0067321.

144. Krupovic M, Makarova KS, Koonin EV. Cellular homologs of the double jelly-roll major capsid proteins clarify the origins of an ancient virus kingdom. Proc Natl Acad Sci U S A. 2022;119:e2120620119.

145. Krupovic M, Yutin N, Koonin E. Evolution of a major virion protein of the giant pandoraviruses from an inactivated bacterial glycoside hydrolase. Virus Evol. 2020;6:veaa059.

146. Krupovic M, Blomberg J, Coffin JM, et al. Ortervirales: new virus order unifying five families of reverse-transcribing viruses. J Virol. 2018;92:e00515-18.

147. Krupovic M, Cvirkaite-Krupovic V, Iranzo J, et al. Viruses of archaea: Structural, functional, environmental and evolutionary genomics. Virus Res. 2018;244:181-193.

148. Krylov DM, Koonin EV. A novel family of predicted retroviral-like aspartyl proteases with a possible key role in eukaryotic cell cycle control. Curr Biol. 2001;11:R584-587.

149. Kvansakul M, Caria S, Hinds MG. The Bcl-2 family in host-virus interactions. Viruses. 2017;9:290.

150. Kyrieleis OJ, Chang J, de la Pena M, et al. Crystal structure of vaccinia virus mRNA capping enzyme provides insights into the mechanism and evolution of the capping apparatus. Structure. 2014;22:452-465.

151. Langland JO, Jacobs BL. The role of the PKR-inhibitory genes, E3L and K3L, in determining vaccinia virus host range. Virology. 2002;299:133-141.

152. Lee BD, Koonin EV. Viroids and viroid-like circular RNAs: do they descend from primordial replicators? Life (Basel). 2022;12:103.

153. Legendre M, Arslan D, Abergel C, et al. Genomics of megavirus and the elusive fourth domain of life. Commun Integr Biol. 2012;5:102-106.

154. Legendre M, Fabre E, Poirot O, et al. Diversity and evolution of the emerging Pandoraviridae family. Nat Commun. 2018;9:2285.

155. Lehmann KC, Gulyaeva A, Zevenhoven-Dobbe JC, et al. Discovery of an essential nucleotidylating activity associated with a newly delineated conserved domain in the RNA polymerase-containing protein of all nidoviruses. Nucleic Acids Res. 2015;43:8416-8434.

156. Leipe DD, Aravind L, Koonin EV. Did DNA replication evolve twice independently? Nucleic Acids Res. 1999;27:3389-3401.

157. Leipe DD, Koonin EV, Aravind L. Evolution and classification of P-loop kinases and related proteins. J Mol Biol. 2003;333:781-815.

158. Li Y, Bondy-Denomy J. Anti-CRISPRs go viral: the infection biology of CRISPR-Cas inhibitors. Cell Host Microbe. 2021;29:704-714.

159. Li C, Debing Y, Jankevicius G, et al. Viral macro domains reverse protein ADP-ribosylation. J Virol. 2016;90:8478-8486.

160. Li G, Liu X, Yang M, et al. Crystal structure of African swine fever virus pS273R protease and implications for inhibitor design. J Virol. 2020;94:e02125-19.

161. Li M, Ye G, Si Y, et al. Structure of the multiple functional domains from coronavirus nonstructural protein 3. Emerg Microbes Infect. 2021;10:66-80.

162. Lin MH, Chang SC, Chiu YC, et al. Structural, biophysical, and biochemical elucidation of the SARS-CoV-2 nonstructural protein 3 macro domain. ACS Infect Dis. 2020;6:2970-2978.

163. Lingel A, Simon B, Izaurralde E, et al. The structure of the flock house virus B2 protein, a viral suppressor of RNA interference, shows a novel mode of double-stranded RNA recognition. EMBO Rep. 2005;6:1149-1155.

164. Liu J, Mushegian A. Displacements of prohead protease genes in the late operons of double-stranded-DNA bacteriophages. J Bacteriol. 2004;186:4369-4375.

165. Liu Y, Xu L, Opalka N, et al. Crystal structure of sTALL-1 reveals a virus-like assembly of TNF family ligands. Cell. 2002;108:383-394.

166. Liu L, Yin M, Wang M, et al. Phage AcrIIA2 DNA mimicry: structural basis of the CRISPR and anti-CRISPR arms race. Mol Cell. 2019;73:611-620 e613.

167. Liu Y, Bisio H, Toner CM, et al. Virus-encoded histone doublets are essential and form nucleosome-like structures. Cell. 2021;184:4237-4250 e4219.

168. Liu Y, Demina TA, Roux S, et al. Diversity, taxonomy, and evolution of archaeal viruses of the class Caudoviricetes. PLoS Biol. 2021;19:e3001442.

169. Loh B, Kuhn A, Leptihn S. The fascinating biology behind phage display: filamentous phage assembly. Mol Microbiol. 2019;111:1132-1138.

170. Longo LM, Jablonska J, Vyas P, et al. On the emergence of P-Loop NTPase and Rossmann enzymes from a Beta-Alpha-Beta ancestral fragment. Elife. 2020;9:e64415.

171. Lu A, Miller LK. Identification of three late expression factor genes within the 33.8- to 43.4-map-unit region of Autographa californica nuclear polyhedrosis virus. J Virol. 1994;68:6710-6718.

172. Lucas WJ. Plant viral movement proteins: agents for cell-to-cell trafficking of viral genomes. Virology. 2006;344:169-184.

173. Luo X, Sanford DG, Bullock PA, et al. Solution structure of the origin DNA-binding domain of SV40 T-antigen. Nat Struct Biol. 1996;3:1034-1039.

174. Mancini EJ, Tuma R. Mechanism of RNA packaging motor. Adv Exp Med Biol. 2012;726:609-629.

175. Mancini EJ, Kainov DE, Grimes JM, et al. Atomic snapshots of an RNA packaging motor reveal conformational changes linking ATP hydrolysis to RNA translocation. Cell. 2004;118:743-755.

176. Marq JB, Hausmann S, Luban J, et al. The double-stranded RNA binding domain of the vaccinia virus E3L protein inhibits both RNA- and DNA-induced activation of interferon beta. J Biol Chem. 2009;284:25471-25478.

177. Melcher U. The '30K' superfamily of viral movement proteins. J Gen Virol. 2000;81:257-266.

178. Mendes A, Kuhn RJ. Alphavirus nucleocapsid packaging and assembly. Viruses. 2018;10:138.

179. Mesyanzhinov VV, Robben J, Grymonprez B, et al. The genome of bacteriophage phiKZ of *Pseudomonas aeruginosa*. J Mol Biol. 2002;317:1-19.

180. Mirkin BG, Fenner TI, Galperin MY, et al. Algorithms for computing parsimonious evolutionary scenarios for genome evolution, the last universal common ancestor and dominance of horizontal gene transfer in the evolution of prokaryotes. BMC Evol Biol. 2003;3:2.

181. Mizuno CM, Guyomar C, Roux S, et al. Numerous cultivated and uncultivated viruses encode ribosomal proteins. Nat Commun. 2019;10:752.

182. Molodtsov V, Murakami KS. Minimalism and functionality: structural lessons from the heterodimeric N4 bacteriophage RNA polymerase II. J Biol Chem. 2018;293: 13616-13625.

183. Monttinen HA, Ravantti JJ, Stuart DI, et al. Automated structural comparisons clarify the phylogeny of the right-hand-shaped polymerases. Mol Biol Evol. 2014;31:2741-2752.

184. Moral-Lopez P, Alvarez E, Redondo N, et al. L protease from foot and mouth disease virus confers eIF2-independent translation for mRNAs bearing picornavirus IRES. FEBS Lett. 2014;588:4053-4059.

185. Morozov SY, Solovyev AG. Triple gene block: modular design of a multifunctional machine for plant virus movement. J Gen Virol. 2003;84:1351-1366.

186. Moss D, Smith GL. Poxviridae: the viruses and their replication. In: Fields Virology. LWW; 2021.

187. Moss B, Shisler JL, Xiang Y, et al. Immune-defense molecules of molluscum contagiosum virus, a human poxvirus. Trends Microbiol. 2000;8:473-477.

188. Murakami KS, Davydova EK, Rothman-Denes LB. X-ray crystal structure of the polymerase domain of the bacteriophage N4 virion RNA polymerase. Proc Natl Acad Sci U S A. 2008;105:5046-5051.

189. Mushegian AR, Elena SF. Evolution of plant virus movement proteins from the 30K superfamily and of their homologs integrated in plant genomes. Virology. 2015;476C:304-315.

190. Mushegian AR, Koonin EV. Cell-to-cell movement of plant viruses. Insights from amino acid sequence comparisons of movement proteins and from analogies with cellular transport systems. Arch Virol. 1993;133:239-257.

191. Mushegian AR, Koonin EV. A minimal gene set for cellular life derived by comparison of complete bacterial genomes. Proc Natl Acad Sci U S A. 1996;93:10268-10273.

192. Nasir A, Romero-Severson E, Claverie JM. Investigating the concept and origin of viruses. Trends Microbiol. 2020;28:959-967.

193. Navarro JA, Pallas V. An update on the intracellular and intercellular trafficking of carmoviruses. Front Plant Sci. 2017;8:1801.

194. Nelson CA, Epperson ML, Singh S, et al. Structural conservation and functional diversity of the poxvirus immune evasion (PIE) domain superfamily. Viruses. 2015;7:4878-4898.

195. Neuvonen M, Ahola T. Differential activities of cellular and viral macro domain proteins in binding of ADP-ribose metabolites. J Mol Biol. 2009;385:212-225.

196. Ng KK, Arnold JJ, Cameron CE. Structure-function relationships among RNA-dependent RNA polymerases. Curr Top Microbiol Immunol. 2008;320:137-156.

197. Ogino T, Green TJ. RNA Synthesis and capping by non-segmented negative strand RNA viral polymerases: lessons from a prototypic virus. Front Microbiol. 2019;10:1490.

198. Olschewski S, Cusack S, Rosenthal M. The Cap-snatching mechanism of bunyaviruses. Trends Microbiol. 2020;28:293-303.

199. Ouzounis CA, Kunin V, Darzentas N, et al. A minimal estimate for the gene content of the last universal common ancestor—exobiology from a terrestrial perspective. Res Microbiol. 2006;157:57-68.

200. Passarelli AL, Todd JW, Miller LK. A baculovirus gene involved in late gene expression predicts a large polypeptide with a conserved motif of RNA polymerases. J Virol. 1994;68:4673-4678.

201. Pavesi A. Origin, evolution and stability of overlapping genes in viruses: a systematic review. Genes (Basel). 2021;12:809.

202. Pavesi A, Magiorkinis G, Karlin DG. Viral proteins originated de novo by overprinting can be identified by codon usage: application to the "gene nursery" of Deltaretroviruses. PLoS Comput Biol. 2013;9:e1003162.

203. Peck KM, Lauring AS. Complexities of viral mutation rates. J Virol. 2018;92:e01031-17.

204. Peremyslov VV, Hagiwara Y, Dolja VV. HSP70 homolog functions in cell-to-cell movement of a plant virus. Proc Natl Acad Sci U S A. 1999;96:14771-14776.

205. Perez-Vargas J, Pereira de Oliveira R, Jacquet S, et al. HDV-like viruses. Viruses. 2021;13:1207.

206. Philosof A, Yutin N, Flores-Uribe J, et al. Novel Abundant oceanic viruses of uncultured marine group II euryarchaeota. Curr Biol. 2017;27:1362-1368.

207. Proulx J, Borgmann K, Park IW. Role of virally-encoded deubiquitinating enzymes in regulation of the virus life cycle. Int J Mol Sci. 2021;22:4438.

208. Pyle JD, Whelan SPJ, Bloyet LM. Structure and function of negative-strand RNA virus polymerase complexes. Enzymes. 2021;50:21-78.

209. Raia P, Delarue M, Sauguet L. An updated structural classification of replicative DNA polymerases. Biochem Soc Trans. 2019;47:239-249.

210. Ramanathan A, Robb GB, Chan SH. mRNA capping: biological functions and applications. Nucleic Acids Res. 2016;44:7511-7526.

211. Rao VB, Feiss M. Mechanisms of DNA packaging by large double-stranded DNA viruses. Annu Rev Virol. 2015;2:351-378.

212. Raoult D, Forterre P. Redefining viruses: lessons from mimivirus. Nat Rev Microbiol. 2008;6:315-319.

213. Ravantti JJ, Martinez-Castillo A, Abrescia NGA. Superimposition of viral protein structures: a means to decipher the phylogenies of viruses. Viruses. 2020;12:1146.

214. Reinisch KM, Nibert ML, Harrison SC. Structure of the reovirus core at 3.6 A resolution. Nature. 2000;404:960-967.

215. Requiao RD, Carneiro RL, Moreira MH, et al. Viruses with different genome types adopt a similar strategy to pack nucleic acids based on positively charged protein domains. Sci Rep. 2020;10:5470.

216. Rissanen I, Grimes JM, Pawlowski A, et al. Bacteriophage P23-77 capsid protein structures reveal the archetype of an ancient branch from a major virus lineage. Structure. 2013;21:718-726.

217. Rodrigues RAL, Arantes TS, Oliveira GP, et al. The complex nature of tupanviruses. Adv Virus Res. 2019;103:135-166.

218. Rolland C, Andreani J, Sahmi-Bounsiar D, et al. Clandestinovirus: a giant virus with chromatin proteins and a potential to manipulate the cell cycle of its host *Vermamoeba vermiformis*. Front Microbiol. 2021;12:715608.

219. Roux S, Krupovic M, Daly RA, et al. Cryptic inoviruses revealed as pervasive in bacteria and archaea across Earth's biomes. Nat Microbiol. 2019;4:1895-1906.

220. Saberi A, Gulyaeva AA, Brubacher JL, et al. A planarian nidovirus expands the limits of RNA genome size. PLoS Pathog. 2018;14:e1007314.

221. Sanjuan R, Domingo-Calap P. Mechanisms of viral mutation. Cell Mol Life Sci. 2016;73:4433-4448.

222. Santos-Perez I, Charro D, Gil-Carton D, et al. Structural basis for assembly of vertical single beta-barrel viruses. Nat Commun. 2019;10:1184.

223. Senkevich TG, Yutin N, Wolf YI, et al. Ancient gene capture and recent gene loss shape the evolution of orthopoxvirus-host interaction genes mBio. 2021;12:e0149521.

224. Sharon I, Alperovitch A, Rohwer F, et al. Photosystem I gene cassettes are present in marine virus genomes. Nature. 2009;461:258-262.

225. Shin J, Jiang F, Liu JJ, et al. Disabling Cas9 by an anti-CRISPR DNA mimic. Sci Adv. 2017;3:e1701620.

226. Shuman S. What messenger RNA capping tells us about eukaryotic evolution. Nat Rev Mol Cell Biol. 2002;3:619-625.

227. Shutt TE, Gray MW. Bacteriophage origins of mitochondrial replication and transcription proteins. Trends Genet. 2006;22:90-95.

228. Silva AM, Cachau RE, Sham HL, et al. Inhibition and catalytic mechanism of HIV-1 aspartic protease. J Mol Biol. 1996;255:321-346.

229. Simmonds P, Becher P, Bukh J, et al. ICTV virus taxonomy profile: Flaviviridae. J Gen Virol. 2017;98:2-3.

230. Sirkis R, Gerst JE, Fass D. Ddi1, a eukaryotic protein with the retroviral protease fold. J Mol Biol. 2006;364:376-387.

231. Sokolova ML, Misovetc I, Severinov KV. Multisubunit RNA polymerases of jumbo bacteriophages. Viruses. 2020;12:1064.

232. Straus SK, Bo HE. Filamentous bacteriophage proteins and assembly. Subcell Biochem. 2018;88:261-279.

233. Strömsten NJ, Bamford DH, Bamford JK. In vitro DNA packaging of PRD1: a common mechanism for internal-membrane viruses. J Mol Biol. 2005;348:617-629.

234. Suhanovsky MM, Teschke CM. Nature's favorite building block: deciphering folding and capsid assembly of proteins with the HK97-fold. Virology. 2015;479-480:487-497.

235. Sullivan MB, Lindell D, Lee JA, et al. Prevalence and evolution of core photosystem II genes in marine cyanobacterial viruses and their hosts. PLoS Biol. 2006;4:e234.

236. Tahirov TH, Makarova KS, Rogozin IB, et al. Evolution of DNA polymerases: an inactivated polymerase-exonuclease module in Pol epsilon and a chimeric origin of eukaryotic polymerases from two classes of archaeal ancestors. Biol Direct. 2009;4:11.

237. Tahirov TH, Temiakov D, Anikin M, et al. Structure of a T7 RNA polymerase elongation complex at 2.9 A resolution. Nature.

2002;420:43-50.

238. Taliansky M, Torrance L, Kalinina NO. Role of plant virus movement proteins. Methods Mol Biol. 2008;451:33-54.

239. te Velthuis AJ. Common and unique features of viral RNA-dependent polymerases. Cell Mol Life Sci. 2014;71:4403-4420.

240. Te Velthuis AJW, Grimes JM, Fodor E. Structural insights into RNA polymerases of negative-sense RNA viruses. Nat Rev Microbiol. 2021;19:303-318.

241. Thomas V, Bertelli C, Collyn F, et al. Lausannevirus, a giant amoebal virus encoding histone doublets. Environ Microbiol. 2011;13:1454-1466.

242. Thomas JA, Weintraub ST, Wu W, et al. Extensive proteolysis of head and inner body proteins by a morphogenetic protease in the giant *Pseudomonas aeruginosa* phage phiKZ. Mol Microbiol. 2012;84:324-339.

243. Torres AA, Albarnaz JD, Bonjardim CA, et al. Multiple Bcl-2 family immunomodulators from vaccinia virus regulate MAPK/AP-1 activation. J Gen Virol. 2016;97:2346-2351.

244. Twarock R, Stockley PG. RNA-mediated virus assembly: mechanisms and consequences for viral evolution and therapy. Annu Rev Biophys. 2019;48:495-514.

245. Ulferts R, Ziebuhr J. Nidovirus ribonucleases: structures and functions in viral replication. RNA Biol. 2011;8:295-304.

246. Valencia-Sanchez MI, Abini-Agbomson S, Wang M, et al. The structure of a virus-encoded nucleosome. Nat Struct Mol Biol. 2021;28:413-417.

247. van den Born E, Omelchenko MV, Bekkelund A, et al. Viral AlkB proteins repair RNA damage by oxidative demethylation. Nucleic Acids Res. 2008;36:5451-5461.

248. Van Etten JL, Agarkova IV, Dunigan DD. Chloroviruses. Viruses. 2019;12:20.

249. Veesler D, Ng TS, Sendamarai AK, et al. Atomic structure of the 75 MDa extremophile Sulfolobus turreted icosahedral virus determined by CryoEM and X-ray crystallography. Proc Natl Acad Sci U S A. 2013;110:5504-5509.

250. Walker AP, Fan H, Keown JR, et al. The SARS-CoV-2 RNA polymerase is a viral RNA capping enzyme. Nucleic Acids Res. 2021;49:13019-13030.

251. Walker JE, Saraste M, Runswick MJ, et al. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. 1982;1:945-951.

252. Wang H, Guo Z, Feng H, et al. Novel Sulfolobus virus with an exceptional capsid architecture. J Virol. 2018;92:e01727-17.

253. Wang HC, Hirono I, Maningas MBB, et al. ICTV virus taxonomy profile: Nimaviridae. J Gen Virol. 2019;100:1053-1054.

254. Webb B, Rakibuzzaman A, Ramamoorthy S. Torque teno viruses in health and disease. Virus Res. 2020;285:198013.

255. Weber JM. Adenain, the adenovirus endoprotease (a review). Acta Microbiol Immunol Hung. 2003;50:95-101.

256. Weigel C, Seitz H. Bacteriophage replication modules. FEMS Microbiol Rev. 2006;30:321-381.

257. Wiegand T, Karambelkar S, Bondy-Denomy J, et al. Structures and strategies of anti-CRISPR-mediated immune suppression. Annu Rev Microbiol. 2020;74:21-37.

258. Wikoff WR, Liljas L, Duda RL, et al. Topologically linked protein rings in the bacteriophage HK97 capsid. Science. 2000;289:2129-2133.

259. Williams TA, Embley TM, Heinz E. Informational gene phylogenies do not support a fourth domain of life for nucleocytoplasmic large DNA viruses. PLoS One. 2011;6:e21080.

260. Wolf YI, Kazlauskas D, Iranzo J, et al. Origins and evolution of the global RNA virome. mBio. 2018;9:e02329-18.

261. Xiong Y, Eickbush TH. Origin and evolution of retroelements based upon their reverse transcriptase sequences. EMBO J. 1990;9:3353-3362.

262. Yang Y, Yang P, Wang N, et al. Architecture of the herpesvirus genome-packaging complex and implications for DNA translocation. Protein Cell. 2020;11:339-351.

263. Yin Y, Fischer D. Identification and investigation of ORFans in the viral world. BMC Genomics. 2008;9:24.

264. Yoshikawa G, Blanc-Mathieu R, Song C, et al. Medusavirus, a novel large DNA virus discovered from hot spring water. J Virol. 2019;93.

265. Yu H, Bruneau RC, Brennan G, et al. Battle Royale: innate recognition of poxviruses and viral immune evasion. Biomedicines. 2021;9:765.

266. Yutin N, Koonin EV. Hidden evolutionary complexity of nucleo-cytoplasmic large DNA viruses of eukaryotes. Virol J. 2012;9:161.

267. Yutin N, Benler S, Shmakov SA, et al. Analysis of metagenome-assembled viral genomes from the human gut reveals diverse putative CrAss-like phages with unique genomic features. Nat Commun. 2021;12(1):1044.

268. Yutin N, Faure G, Koonin EV, et al. Chordopoxvirus protein F12 implicated in enveloped virion morphogenesis is an inactivated DNA

polymerase. Biol Direct. 2014;9:22.

269. Yutin N, Makarova KS, Gussow AB, et al. Discovery of an expansive bacteriophage family that includes the most abundant viruses from the human gut. Nat Microbiol. 2018;3:38-46.

270. Yutin N, Wolf YI, Koonin EV. Origin of giant viruses from smaller DNA viruses not from a fourth domain of cellular life. Virology. 2014;466-467:38-52.

271. Zhang K, Law YS, Law MCY, et al. Structural insights into viral RNA capping and plasma membrane targeting by Chikungunya virus nonstructural protein 1. Cell Host Microbe. 2021;29:757-764 e753.

272. Zuhlsdorf M, Hinrichs W. Assemblins as maturational proteases in herpesviruses. J Gen Virol. 2017;98:1969-1984.

CHAPTER 13

Epidemiology

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Epidemiology

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Acknowledgments

Epidemiology

Epidemiology is the science of measuring patterns of health and disease and the impact of interventions across time, space, and populations. Basic concepts of infectious disease epidemiology, including incubation period, reproductive number, and herd immunity, entered public discourse during the SARS-CoV-2 pandemic and are no longer the special purview of epidemiologists, public health practitioners, infectious disease specialists, and virologists. Epidemiologic patterns of viral diseases, such as smallpox and measles, were recognized and characterized long before their causal agents were discovered, and viral epidemiology was one of the first aspects of the science of virology to be developed. Evolving insights into the molecular biology, genomics, pathogenesis, and host immune responses to viruses have provided an increasingly rational basis for understanding the epidemiology of viruses.

Historically, epidemiology has its roots in infectious diseases, with methods for studying chronic diseases such as diabetes and cardiovascular diseases maturing after World War II. What distinguishes infectious disease epidemiology from chronic disease epidemiology is that the former accounts for *dependent happenings*, a term introduced by Ronald Ross, in which the incidence of a disease depends on its prevalence within a population.[81] Traditional statistical methods that are used in epidemiologic studies to infer relationships between exposure and disease outcome assume that the outcome in one individual is independent of the outcome in other individuals, an assumption that is invalid for communicable viral infections.[66] This dependent nature of viral infections is what makes their epidemiology so dynamic and complex, and their study so fascinating.

Basic Definitions and Methods of Epidemiology

Measures of Disease Burden

The quantification of disease occurrence is the cardinal feature of epidemiology. To accomplish this, the concept of rates was introduced, and rates have become the basic coinage of epidemiology (Fig. 13.1). Rates are fractions in which the numerator is the number of cases of disease and the denominator is a measure of the population. The *incidence rate* (also called the *attack rate* for acute infectious diseases) is used to quantify the number of new infections. A population and a time frame are defined, and the number of new cases in that population during that interval of time is enumerated. The denominator includes both the size of population and the time frame of measurement and is often expressed as person-years (or any other standard interval of time: eg, “thousand person-years” or “hundred person-weeks”). The incidence rate is then expressed as “cases per thousand person-years” or a similar term. The time element may not be explicitly stated, in which case the reader must then determine by context the time frame used.

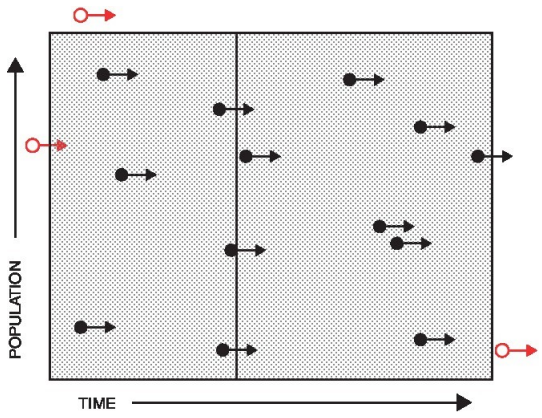

FIGURE 13.1 **Computation of incidence rate and prevalence ratio**. The *shaded area* defines a population and a time frame that can be expressed as person-time units. Cases of disease are indicated by *circles* (placed according to the date of their onset) and *arrows* for duration of illness. *Solid black circles* would be counted for computation of incidence, whereas *open red circles* would be excluded because they had onset outside the designated time frame or were resident outside the population boundaries. Incidence equals the number of cases divided by population-time units. Prevalence would be determined by a *single vertical line* across the hatched area: cases active at that time point would be divided by the size of the population to compute the prevalence.

Prevalence is not a rate but a proportion and refers to the total number of cases present in a defined population within a specified time interval. Thus, the numerator in prevalence includes not just new cases but cases carried forward from the period prior to the specified time interval, a distinction more important for chronic than acute viral infections. When a point or period prevalence is computed, a particular narrow time point or frame is selected (eg, 1 month), and the average population size within that time frame constitutes the denominator. All prevalent cases during that period constitute the numerator. Prevalence is expressed as a ratio, such as “cases per million persons.” Note that there is no time unit in this ratio.

In calculating incidence and prevalence, persons counted in the denominator should be at risk to be a case in the numerator if infected. For instance, if incidence is computed for the population of Baltimore for 2022, a case hospitalized in the city but resident outside the city would not be counted. A case with onset in December 2021 but still ill or infected in 2022 would not be counted as an incident case but would be a prevalent case. Immune individuals are often included in the denominator because their measurement is not possible without additional information, such as from serological surveys. The *force of infection* is the rate at which susceptible individuals are infected.

The relationship between incidence and prevalence depends on the average duration of disease, and changes in incidence and prevalence may be divergent. A control program for human immunodeficiency virus (HIV) infection, for example, could decrease HIV incidence through effective preventative measures but increase the prevalence of HIV infection through access to antiretroviral therapy and improved survival.

Data Sources for Measuring Disease Burden

In practice, accurate data collection for the computation of incidence and prevalence is often a major undertaking, whereas the computations are relatively simple. Such data collection requires access to populations (eg, at health care facilities, the workplace, or at home) and accurate diagnostic tests. Epidemiologists must frequently work with incomplete and inaccurate information, including nonrepresentative study populations and diagnostic tests with imperfect sensitivity and specificity. In some settings, denominator (population) information is accurate and the challenge is measuring the numerator (cases). But in other settings, accurate population data may be unavailable, further hindering efforts to estimate incidence and prevalence.

For infectious diseases, several sources of case data are routinely available. *Passive surveillance* denotes the reporting of disease by health care workers. By law, many viral diseases are designated as "reportable," and in theory, the reporting of all cases is required. Cases are reported by practicing physicians, hospitals, or laboratories to local health jurisdictions, which in turn relay reports to a central state office where they are transmitted to a national center (eg, the U.S. Centers for Disease Control and Prevention). The weakest link is the initial report; in practice, only a small fraction of cases of many common viral diseases present for care, are correctly identified, and are reported to the national center (reflecting the *reporting efficiency*). However, if the proportion of cases reported is consistent between geographic locales and between years, these reports may be used to monitor trends. Consistency of reporting efficiency is important because the proportion of cases reported may increase if the number of cases decreases. Changes in the case definition, for example, also may result in spurious changes in surveillance data. With SARS-CoV-2, increasing availability of home testing with rapid diagnostic tests led to under-reporting.[77] Reporting of certain rare or serious diseases, such as acquired immunodeficiency syndrome (AIDS), is often close to 100%, and reported cases can be used with greater confidence for calculation of rates.

With increasing interest in the pandemic spread of respiratory viruses and the emergence of new zoonotic viral pathogens, passive global disease surveillance networks have been established to provide data on disease incidence and transmission in real time, including the Program for Monitoring Emerging Diseases (ProMED) and the World Health Organization's Global Outbreak Alert and Response Network (GOARN). Such surveillance systems will likely be strengthened in response to the SARS-CoV-2 pandemic but are only as good as the timeliness and accuracy of reporting. Countries should be encouraged to report outbreaks as soon as they are identified.

Active surveillance through epidemic investigation is the traditional approach to collecting information on disease outbreaks. Such investigations are usually initiated by public health authorities but may be instigated by health care workers or patient families. The investigation is tailored to the situation and resources available. The goals of outbreak investigations are to (1) establish a case definition by classifying the illness and determining the causative organism; (2) assess the extent of the outbreak and its economic and health impact; (3) mitigate the outbreak and prevent future outbreaks; and (4) inform accurate and timely health communications with the public. The first recognition of a new disease or isolation of a previously unknown virus results from an epidemic investigation.

Serological surveys may be used to detect the immunologic footprints that a virus leaves in a population. Serosurveys are particularly useful for viruses because most viral infections leave an imprint on all infected individuals—that is, the presence of virus-specific immunoglobulin G (IgG) antibodies that can persist for years and in some cases, such as measles-specific IgG, many decades. Incident viral infections can be identified using assays for immunoglobulin M (IgM) antibodies or antibody avidity, whereas IgG antibodies indicate prior infection or vaccination. Because many viruses cause asymptomatic infections or nondescript illnesses in addition to diagnosable diseases, serological surveys identify inapparent as well as apparent infections. Serological surveys have been critical to understanding the extent of SARS-CoV-2 infection,[6] and repeated serological surveys can be used to track changes in cumulative exposure by age and geographic location over the course of the epidemic as well as to evaluate the impact of nonpharmaceutical interventions. However, household-based serosurveys can be labor intensive and expensive and results are not often available in time for prompt public health decision-making. The use of residual specimens from blood banks or health care facilities can lower the costs associated with serosurveys but may be less representative of the general population.

Cohort and Case-Control Study Designs

Epidemiological investigations not only provide measures of disease burden but also of risk factors for disease. To assess risk factors associated with specific viral infections, comparisons between those with and without the infection are necessary. Different types of study designs are used to make these comparisons, depending on the research question, viral infection, outcomes and exposures of interest, study population, and available resources. In general, epidemiologists attempt to test the hypothesis that one or more population variables influence the occurrence of a specified disease and, if so, to quantify this effect and estimate confidence limits. There are two basic observational designs used for such studies, *cohort* and *case-control* studies, shown in Figure 13.2, although other study designs are also used.

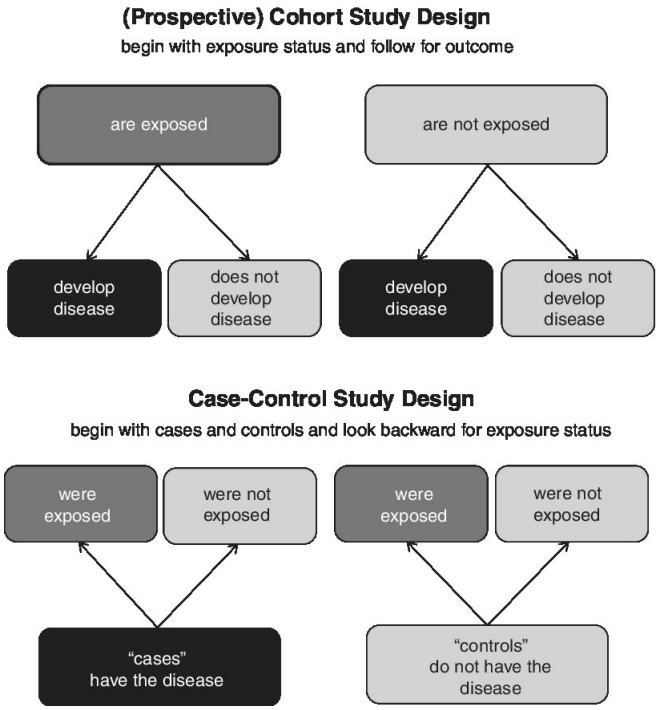

FIGURE 13.2 **Schematic of two basic epidemiological study designs**. **Upper panel**.(Prospective) cohort study design. **Lower panel**. Case-control study design.

Cohort Studies

In a cohort study, the population falls into one of two groups, one with and one without a specified exposure or attribute. Both groups are followed and the *incidence* of the disease under study is measured. Incidence rates for both groups are then computed and compared. In a prospective cohort study, the exposure is measured and participants are then followed into the future. In a retrospective cohort study, the exposure is recorded from past measurements and the disease outcome determined up to the present time. A hypothetical example is shown in Table 13.1. In this example, the population attribute is immunization against a specified viral disease, and the attack rate in the immunized population is one-third the rate in the unimmunized control group. The *relative risk* (RR) associated with immunization is 0.33, and vaccine efficacy is 67%, derived from the equation (1 − RR) × 100. The important assumption underlying the validity of such a study is that the immunized and unimmunized groups are at equal risk of disease. For various reasons, this assumption may not be correct. Differences in the risk of disease between two study groups may be minimized by random allocation of the exposure (in this case, the vaccine) when that is deemed ethical, as done in randomized clinical trials. However, random allocation of exposure is not possible unless the study is of an intervention. Stratifying each study arm according to population characteristics such as age, race, and socioeconomic status and then comparing rates for each subgroup can be used to test the assumption that both groups are at equal risk of disease. More commonly, these variables are adjusted for in a regression analysis.

TABLE 13.1 Hypothetical data to illustrate computations for a cohort and a case-control study of vaccine efficacy

Group	2012 Cases	Population	Incident Cases per 100 000	Relative Risk
Cohort study[a]				
Vaccinated	100	1 000 000	10	0.33
Unvaccinated	900	3 000 000	30	
Group	**Cases**	**Controls**	**Odds**	**Odds Ratio**
Case-control study[b]				
Vaccinated	10	25	10/90	0.33
Unvaccinated	90	75	25/75	

Vaccine efficacy = 1 − relative risk = 0.67.

[a]Cohort study: Two populations, vaccinated and not vaccinated, are followed for 1 year, and incident cases occurring in each group are recorded. Risk for each exposure group is calculated, and the ratio of risks gives the relative risk for those who were vaccinated. In this instance, relative risk is lower for those with than without the attribute (immunization). The validity of this design depends on the assumption that vaccinated and not vaccinated groups would be at equal risk except for the attribute under study.

[b]Case-control study: A group of 100 cases and 100 controls are randomly picked to be representative of the groups from which they are drawn. Subjects in each group are classified as vaccinated or not vaccinated. The odds of a case being exposed (vaccinated) and the odds of the control being exposed are used to compute the odds ratio, which provides an estimate of the relative risk. The validity of the case-control design depends on two assumptions: (1) the case and control groups are representative of the larger groups from which they are drawn and (2) the number of cases is very small (<1/10) relative to the total population.

More complex study designs are used to measure indirect effects of vaccines or other interventions. Indirect effects are when protection is conferred on those who do not receive the vaccine or intervention and is a consequence of the dependent nature of viral infections as described above.[44] Such indirect effects include protection of nonvaccinated individuals through decreased transmission from vaccinated individuals or the decreased probability of an infectious individual encountering a susceptible individual, referred to as herd immunity.[26] Cluster randomized trials—for example, studies in which communities rather than individuals are randomized to receive the intervention—allow measurement of the indirect effects of vaccines by comparing the risk of disease outcomes in nonvaccinated individuals residing in intervention and control communities.[43] If an indirect effect is present, the incidence of disease will be lower among nonvaccinated individuals in the intervention community (eg, the community in which the vaccine was administered) compared to nonvaccinated individuals in the control community with no vaccination.

Case-Control Studies

Cohort studies usually require extensive resources and time because they necessitate the enrollment of large numbers of subjects who must be followed for a period of months or years. The less frequent the expectation of disease, the larger the population or longer the follow-up needed. The costs of prospective cohort studies can be great, limiting them to situations such as the evaluation of a new drug or vaccine, as illustrated in Table 13.2. In contrast, case-control studies can be more cost-effective, particularly if the disease outcome is rare, because they involve smaller numbers of subjects and do not require longitudinal follow-up. Table 13.1 shows a simplified version of such a study. In this instance, 100 cases of the study disease and 100 unaffected control subjects are identified and classified according to the exposure or attribute (vaccination) under study. For both cases and controls, the odds are computed and an odds ratio is estimated. Because, under certain assumptions, the odds ratios have the same relationship as the rates computed in the prospective study shown in the upper half of Table 13.1, the relative risk can be estimated as the ratio of the two odds (0.4/1.2 = 0.33). Two assumptions underlie the validity of the case-control design and analysis, specifically the use of the odds ratio as an estimate of relative risk: the two samples (cases and controls) must be representative of the population from which they are drawn, and the incidence of disease must be low (eg, cases comprise <10% of the total population) so that the odds ratio more closely approximates the relative risk. Because case-control studies are particularly useful to study risk factors for rare diseases, the second assumption is usually correct. The main challenge in designing case-control studies is the choice of valid and representative control groups. Another challenge is accurately measuring the risk of exposure by avoiding information biases, as can occur when cases are more likely to recall or report an exposure than controls.

TABLE 13.2 The 1954 field trial of poliomyelitis vaccine: comparison of attack rates for vaccinated and unvaccinated children[a]

Study Area	Vaccination Group	Population	Paralytic Cases	Attack Rate per 100 000	Estimated Vaccine Efficacy
Placebo areas	Vaccinated Placebo Not inoculated	201 000 201 000 339 000	33 115 121	16 57 36	72%
Observed areas	Vaccinated Controls Not inoculated	222 000 725 000 124 000	38 330 43	17 46 35	63%

[a]Vaccine was administered in the spring of 1954, and children were followed prospectively through the summer poliomyelitis season. Placebo area included volunteers randomized into vaccinated or received placebo inoculations, and nonvolunteers (not inoculated). Observed areas were divided into second-grade volunteers (vaccinated), first and third graders (controls), and second-grade nonvolunteers (not inoculated).

From Francis T Jr, Napier JA, Voight RB, et al. *Evaluation of the 1954 Field Trial of Poliomyelitis Vaccine. Final Report*. University of Michigan; 1957.

A type of case-control study increasingly used to measure vaccine effectiveness is the test-negative design in which the same case definition is used for cases and controls but cases are defined by a positive laboratory test and controls by a negative laboratory test. The test-negative design can be more efficient as both cases and controls are enrolled at the same study site using the same case definition and reduces potential selection biases by ensuring both cases and controls are from the same source population.

Descriptive Epidemiology

Descriptions of epidemic or endemic diseases typically include the parameters person, place, and time. The collection and visualization of these descriptive data is a necessary first step in understanding the epidemiologic processes leading to occurrence, distribution, and course of an epidemic. This simple, systematic approach is a surprisingly powerful tool in analysis.

Person

In tabulating cases of viral infection, the epidemiologist looks for features that distinguish affected persons from the general population to identify risk factors for infection and disease. Clues may exist in demographic features and behavioral characteristics, such as age, sex, gender, race/ethnicity, occupation, residence, or any aspect of personal behavior. Such variables are frequently the most important initial step in understanding an outbreak and designing control measures.

The age distribution of viral infection reflects differences in risk. For many endemic human viruses, the cumulative incidence of infection reaches 100% of the population. In such instances, disease is confined to children or to children and young adults because older individuals are immune because of prior infection. Poliomyelitis prior to the introduction of vaccine is such an example, and differences in age distribution of disease in different regions or in the same population in different eras reflect differing transmission rates.[69] Where this enterovirus was transmitted readily, cases were confined to young children. However, in countries with access to clean water and sanitation, infection may be delayed so that poliomyelitis occurred up to age 30 years or older.[68]

Age-specific differences in case infection ratios can be a key determinant of epidemiologic patterns of disease. In some instances, infants experience much more severe disease than do adults. An important historical example is the outbreak of measles that occurred in the Faroe Islands in 1846,[73] long before measles was known to be caused by a virus. Because measles had not occurred in this isolated site for more than 50 years, women of childbearing age were seronegative, and their infants lacked maternal antibody. During the epidemic, the case fatality ratio was about 20% in infants and below 1% in children and young adults. On the other hand, hepatitis B virus causes inapparent but persistent infection in infants but acute liver disease followed by immunity in adults. In low- and some middle-income countries, infections are frequently transmitted from persistently infected mothers to their newborns, whereas infection in high-income countries is mainly transmitted to older children and adults. These differences account for the paradox that the attack rates for hepatitis B virus are higher in high-income countries, whereas cumulative infection rates are much higher and the virus carrier state is more frequent in low- and middle-income countries.

In addition to identifying individual characteristics associated with viral infection or disease, epidemiologists increasingly are interested in transmission networks critical to the dynamics of viral infections within populations.[54] Network theory and analysis is a complex subject with a long history in mathematics and sociology but has been developed by infectious disease epidemiologists, in part because of the ability to characterize complex social networks, such as large-scale social and sexual networks.[20] The epidemiologic study of social networks is facilitated by unique sampling strategies, including snowball sampling or respondent-driven sampling in which study participants are asked to recruit additional participants among their social contacts. For example, concurrent partnerships (as opposed to serial monogamy) can amplify the spread of HIV and other sexually transmitted infections.[60] Social networks were shown to affect transmission of 2009 H1N1 influenza virus and were responsible for cyclical patterns of transmission between the school, community, and household.[9] For SARS-CoV-2, social networks drove transmission patterns, including superspreading events.[13]

Place

Historically, mapping the distribution of disease preceded the development of epidemiology and became more significant with the introduction of place-specific rates. A famous example is John Snow's mapping of cholera cases during the Broad Street outbreak in London. Mapping spatial patterns of disease distribution remains one of the most powerful aspects of descriptive epidemiology and has been enhanced by remote sensing technologies[80] and sophisticated spatial analysis tools, including patch and network models.[79]

Spatial patterns of viral infections are not static but display complex dynamics, one of which is traveling waves of transmission. Measles virus transmission was shown to exhibit spatiotemporal traveling wave patterns in England and Wales, with regional movement from large cities to small towns.[38] The incidence of dengue hemorrhagic fever, a mosquito-borne disease caused by dengue virus, was characterized in Thailand by a spatiotemporal traveling wave emanating from the capital, Bangkok.[18] The spatiotemporal dynamics of rotavirus infection in the United States prior to the introduction of rotavirus vaccine, which typically started in the Southwest in late fall and ended in the Northeast 3 months later, was explained by spatiotemporal variation in birth rates and thus the introduction of susceptible hosts.[75]

These spatial patterns of viral infections are determined in part by human mobility patterns. Infectious disease epidemiologists use a wide array of tools to measure and characterize human movement patterns, including reported travel histories, GPS loggers worn by individuals, air traffic patterns, and mobile cell phone data.[91]

Time

Many acute viral infections exhibit striking seasonal patterns in incidence.[30] In general, three types of mechanisms have been hypothesized to explain the seasonality of viral infections. First are seasonal cycles in host resistance to infection, including seasonal differences in vitamin D levels.[21] The second involves changes in host behavior and contact patterns, particularly the role of schools as shown for measles virus.[28] Third is the role of climatic factors, particularly temperature and humidity. In temperate climates, some viral infections peak in the winter, whereas others peak in the summer. In general, respiratory infections spread more readily in the winter, although they may peak at different times. Conversely, the incidence of enteric viral infections peak in the summer, although rotaviruses are a striking exception. Arbovirus infections are confined to the summer months when their vectors are active. In the tropics, seasonal patterns of viral infections differ from patterns in temperate climates. Influenza virus transmission in the tropics can be either seasonal or relatively constant throughout the year.[87] Strong seasonal transmission of measles virus in sub-Saharan Africa generates large, irregular measles outbreaks and is an example of nonlinear, chaotic viral dynamics.[25]

Basic Concepts of Infectious Disease Epidemiology

Infectious disease epidemiology, including the epidemiology of viral infections, has its own set of basic concepts and methods, in part due to the dependent nature of communicable diseases.

Susceptibility and Immunity

Some acute viral infections confer lifelong immunity, such as measles and rubella.[7] Upon re-exposure after initial infection, there is often reinfection but with minimal virus replication and an anamnestic immune response. Such reinfections are usually covert and almost never severe, resulting in minimal or no shedding of infectious virus. For certain viruses, such as polioviruses or rhinoviruses, immunity is type specific and confers little protection against exposure to a different serotype. Viral variants, such as seasonal influenza A viruses or the omicron variant of SARS-CoV-2, also can escape immunity conferred by prior infection or vaccination with different variants.[14] Susceptibility may not be present immediately after birth. IgG antibodies are actively transported across the placenta, conferring protective immunity to neonates and young infants. For such viral infections, individuals move from a protected state to a susceptible one in the first year of life. Immunity can then be conferred by vaccination or infection.

These simple facts have profound implications for viral epidemiology. For epidemiologic purposes, a population may be divided into three groups: susceptible, infectious, and immune. For some viral infections, persons infected at any time in the past may be considered immune (recovered) and are protected from reinfection and unable to act as links in a transmission chain, whereas susceptible individuals can be

infected, become infectious, and experience disease. For other viral infections, such as the coronaviruses that cause the common cold, immune or recovered individuals may lose protective immunity and reenter the susceptible group. This compartmentalization of the population is the basis for simple SIR (susceptible, infectious, recovered) models of virus transmission[2] that can explain in part the periodicity ("waves") in incidence of many viral infections as the outcome of the buildup and decline of susceptible individuals.

Persistent viral infections differ from acute viral infections because the infected individual may be capable of transmitting the virus over many years and may develop disease at any time during virus persistence. For persistent viruses, the population may be divided into uninfected susceptibles and immune-but-infectious persons who carry serological markers of prior infection but who are still capable of acting as links in the chain of infection. Important examples are hepatitis B virus, HIV, and several herpesviruses (herpes simplex virus, varicella-zoster virus, and cytomegalovirus) that establish latent infection capable of reactivation.

Parameters That Determine Incidence

For acute viral infections, the following three parameters determine incidence: the proportion of the population susceptible, the proportion of the population that is infectious, and the rate of contact between susceptible and infectious individuals, with *contact* defined as an encounter sufficient for transmission. These three parameters constitute the core of dynamic infectious disease models as described below. The proportion of infected persons who become ill—the case infection ratio—determines the proportion of infected persons detected through case surveillance. This ratio can be high for some infections, such as measles and Ebola virus disease, but low for others, including polio. Table 13.3 shows estimated case infection ratios for five viral infections. Asymptomatic infections hindered SARS-CoV-2 surveillance and led to underestimates of the extent of transmission.

TABLE 13.3 Estimated case infection and case fatality ratios for selected viral infections

Virus	Cases per 100 Infections	Fatalities per 100 Cases	Comment
Smallpox	>95 >95	<1 25	Variola minor Variola major
Measles	>95 >95	0.1 2	United States, 1970 Africa, 1988
Poliomyelitis	<1	5	United States, 1955
Hepatitis B	50 <1	3 <0.1	United States, adults Taiwan, infants
Ebola	high high	42 71	DRC, 2020, Zaire ebolavirus Uganda, 2012, Sudan ebolavirus

Proportion Susceptible

When a population is exposed to an infectious individual with a specific virus, the susceptible part of the population will determine viral spread and account for all new cases. The proportion susceptible will reflect the history of infection with the specific virus in the population, the history of immunization for vaccine-preventable viral infections, and the duration of protective immunity following infection and vaccination. As we saw with SARS-Cov-2, states of susceptibility and protection are not all or none and one must be explicit about the timeframe and outcome of interest: susceptible to infection, disease, hospitalization, or death. For infections that confer life-long immunity, new susceptible individuals largely enter the population through births, and thus the birth rate is a key driver of viral transmission dynamics.

Proportion Infectious

The proportion of susceptibles who become infected, and subsequently infectious, during a fixed period (eg, year or season) can vary widely, depending on the dynamics of transmission, which is influenced by the density of susceptibles and contact patterns between susceptible and infectious individuals.

Rate of Contact Between Susceptible and Infectious Individuals

The rate of contact between susceptible and infectious individuals determines the dynamics of viral spread within a population. This rate depends on the mode of transmission (eg, contact, oral, airborne, or vector-borne), age-specific contact patterns or networks between individuals, and the distribution of susceptible and infectious individuals within a population. Spatial clustering of susceptible individuals can result in outbreaks despite a high proportion of immune individuals within the population.[89] Thus, understanding spatial heterogeneities in susceptibility is critical to understanding outbreak risk.

Case Infection and Fatality Ratios

The proportion of infections resulting in overt disease—the *pathogenicity* of the organism—is characteristic for each virus and is strikingly different for different agents. One of the most important early advances in viral epidemiology was the identification of inapparent or subclinical

infections and the insight that most infections caused by some viruses were asymptomatic. The relative frequency of subclinical infections is expressed as the *case infection ratio*—that is, the number of clinical cases per 100 infections. The lethality of disease is a different parameter that represents the *virulence* of the organism and is designated as the *case fatality ratio*. The case fatality ratio is the number of deaths attributable to an infection per 100 cases. Table 13.3 lists selected common viral infections and shows estimated case infection ratios and case fatality ratios for each. It is noteworthy that there is no regular relationship between the case infection ratio and severity of illness (ie, between pathogenicity and virulence). For instance, measles has a very high case infection ratio (>95:100) but a low case fatality ratio in settings with low levels of undernutrition and high access to care (<1:1000 in the United States). Conversely, poliomyelitis has a low case infection ratio (<1:100) but a higher case fatality ratio (~5:100). Determining the case infection ratio is an important objective of outbreak investigations of novel pathogens, such as SARS-CoV-2, as individuals with asymptomatic infection or mild illness can be important drivers of transmission. Typically, early in an outbreak, only cases that come to clinical attention are counted as incident cases, resulting in an underestimate of the true incidence. Accurate measurement of subclinical infection requires population-based surveys with laboratory confirmation (eg, serological surveys).

Incubation, Latent, and Infectious Periods

The course of infection in a single individual can be divided into several periods (Fig. 13.3).[27] The interval from acquisition of infection to onset of illness is the *incubation period*. Note that "onset of illness" must be explicitly defined for each disease and is often measured by the first day on which pathognomonic signs or symptoms are reported, such as a rash. The interval from acquisition of infection to onset of infectiousness is the *latent period*. For most viral diseases, the latent period is shorter than the incubation period. Consequently, the infected individual begins to shed virus prior to the onset of illness (smallpox was a notable exception) and continues to shed during and sometimes after recovery from the acute illness. The effectiveness of isolation measures is reduced when the latent period is significantly shorter than the incubation period as the infected individual is infectious prior to the onset of symptoms. The period during which the infected person is potentially infectious for others is the *infectious period*.

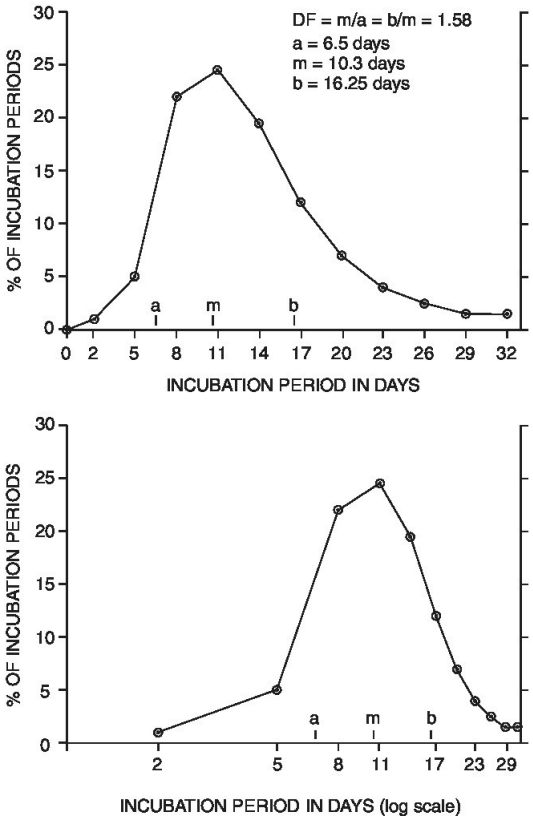

FIGURE 13.3 **Log-normal distribution of incubation periods**. **Upper panel**. Incubation period distribution is shown for poliomyelitis; there is a right-hand tail. **Lower panel**. The same incubation periods when plotted on a logarithmic scale. Logarithmic incubation period approximates a normal distribution, and the standard deviation may be computed (on the logarithmic scale). The dispersion factor (*DF*), a measure of the variation of incubation periods, is the antilogarithm of the standard deviation. The mean (*m*) is shown, together with the standard deviation (points *a* and *b* on the graph). The log DF equals the interval (log *m* to log *a*) or the interval (log *b* to log *m*). In this instance, *m* is about 10.3 days, *a* is 6.5 days, *b* is 16.25 days, and DF is 1.58—that is, about two-thirds of the incubation periods fall into the range (1.58 × mean) to (mean/1.58). (Data from Aycock WL, Luther EH. The incubation period of poliomyelitis. *J Prev Med*. 1929;3:103-120; Casey AE. The incubation period in epidemic poliomyelitis. *JAMA*. 1942;120:805-807; Horstmann DM, Paul JR. The incubation period in human poliomyelitis and its implications. *JAMA*. 1947;135:11-14; Nathanson N, Langmuir AD. The Cutter incident. *Am J Hyg*. 1963;78:16-28; Sartwell PE. The incubation period of poliomyelitis. *Am J Public Health Nations Health*. 1952;42:1403-1408.)

Incubation periods for any viral disease vary around the mean, as illustrated in Figure 13.4. When a frequency distribution of individual incubation periods is plotted, it usually has a longer tail at the high end of the distribution (right skewed). If the frequency is plotted against the logarithm of time, the data often approximate a normal distribution.[82] This log-normal distribution is characteristic of the incubation periods of many viral infections.[52]

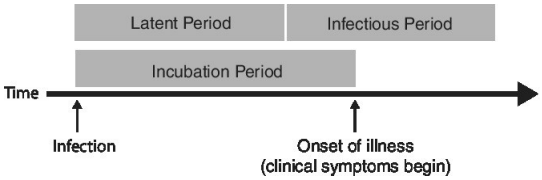

FIGURE 13.4 **Schematic of incubation, latent, and infectious periods**. The relative duration of the periods differs by specific viral infection. In this schematic, the latent period is shorter than the incubation period, implying an infected individual is infectious prior to the onset of symptoms.

The duration of the infectious period is one of the most important determinants of viral transmission dynamics. Acute viral infections are characterized by relatively short infectious periods and excretion of high titers of infectious virus over a short time is necessary to efficiently transmit the virus to susceptible hosts. Examples include measles, smallpox, and influenza viruses. In contrast, viruses with long infectious periods resulting from persistent infection do not require such highly efficient modes of transmission. Examples include hepatitis B virus and HIV. Hepatitis B virus is transmitted to susceptible household contacts at a frequency of <1 per 100 person-years exposure, and HIV is transmitted to sexual contacts at a rate of ~1 transmission per 100 to 1000 contact episodes, with the risk of transmission directly correlated with HIV viral load.[35] Despite inefficient transmission, the number of new infections caused by a persistently infected individual may be high because of the long infectious period. Viral infections can also be persistent due to intermittent periods of activation and latency, such as the herpesviruses (eg, herpes zoster). Thus, an individual may be infected in childhood and transmit infection 50 years later during a recrudescence.

Generation Time and Serial Interval

The average period between infection of an individual and transmission to others is the *generation time* and is primarily determined by the duration of the infectious and latent periods. From studies of selected outbreaks, the average interval between onsets of successive waves of cases (called the *serial interval*) has been determined, and the generation time is often assumed to be equal to this interval as the best approximation available. This is because it is often impossible to know the exact time of infection. These relationships are shown in Figure 13.5 for a typical acute viral infection. Generation time and transmissibility determine the kinetics of the infection. A virus with a relatively short generation time, such as influenza virus, can spread more rapidly than a virus such as poliovirus despite its lower transmissibility (R_0 for influenza virus is ~2 compared to 5-7 for polioviruses).

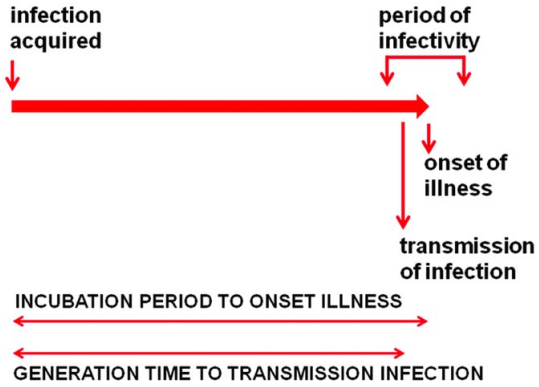

FIGURE 13.5 **Incubation period and generation time**. Incubation period is the interval between acquisition of infection and onset of illness, whereas generation time is the interval between acquisition of infection and transmission to another person. This diagram shows the mean for each parameter; in practice, there is a spread around this mean.

Transmission of Viruses

There are two major patterns of transmission into which viruses may be classified: viruses maintained in a single species and viruses that alternately infect multiple host species. Among multi-host systems, a further distinction important for human hosts are viruses for which transmission is maintained by humans and those in which human cases are sporadic and a dead-end for transmission. There are some viruses that, depending on the setting (eg, urban vs rural), can be both. Examples of these patterns are shown in Table 13.4.

TABLE 13.4 Major transmission patterns of viral infections of humans

Host and Virus Type	Human Transmission Pattern	Maintenance Cycle	Example
Single-host human viruses	Human to human	Human to human	Measles virus Hepatitis A virus Human immunodeficiency virus
Multi-host zoonotic viruses	Animal to human	Animal to animal	Rabies virus Hantavirus Henipaviruses
Multi-host zoonotic viruses	Animal to human or human to human	Animal to animal	Ebolavirus Nipah virus Monkeypox virus
Multi-host zoonotic and vector transmitted viruses	Vector to human	Vector to animal	Japanese encephalitis virus Tick-borne encephalitis virus Yellow fever virus
Multi-host vector transmitted viruses	Vector to human	Vector to human	Dengue virus Yellow fever virus in urban settings Zika virus in urban settings

Viruses Maintained in a Single Host Species

Viruses maintained within a single host population have characteristic modes of transmission: contact, respiratory, oral, or perinatal. Contact transmission includes both direct and indirect contact with infectious individuals, for example, directly through skin or sexual contact or indirectly through infected fomites, blood, or body fluids. Respiratory transmission results from the inhalation of contaminated air, including large respiratory droplets that fall out of the air quickly over short distances and small airborne particles that can remain suspended in the air for several hours. Oral transmission occurs by ingesting contaminated foods or liquids. This includes viruses primarily reliant on the fecal-oral route to maintain transmission, such as rotavirus and polioviruses. Contact, airborne, and oral transmission are horizontal modes. Perinatal transmission denotes transmission from a mother to offspring and is a form of vertical transmission. Perinatal transmission can occur by passage of virus across the placenta, during birth, or from drinking colostrum or breast milk. Germline transmission, another type of vertical transmission, occurs with certain animal retroviruses such as murine leukemia virus, which are transmitted as an integrated provirus that may be transcribed into infectious virus. Although retroviral sequences are carried in the human genome, there are presently no proven instances where these have been shown to function as infectious virions in humans. Selected examples of horizontal and vertical transmission are listed in Table 13.5.

TABLE 13.5 Transmission mechanisms of human viruses maintained by the person-to-person route

General Category	Transmission Mechanism	Examples
Horizontal	Contact	
	Direct	Human papilloma virus, HIV
	Indirect	Influenza virus, hepatitis A virus
	Airborne	SARS-CoV-2, influenza virus
	Oral	Rotavirus, norovirus
Vertical	Perinatal	Rubella virus, cytomegalovirus,
	Via placenta	Zika virus, HIV
	Via maternal parturition	Rubella virus, cytomegalovirus, Zika virus, HIV
	Via maternal breast milk	Herpes simplex virus, hepatitis B virus, HIV

Viruses Maintained in Multiple Host Species

In addition to the four modes of transmission characterized for single-host systems, multi-host systems also include vector transmission. Vector transmission requires a secondary host (ie, vector) to spread the virus. Vector-borne viral infections are usually maintained through continuously cycling between the nonvertebrate host vectors (ie, arthropods such as mosquitos, ticks, sandflies) and vertebrate hosts. In most instances, the virus replicates optimally in a single species or in a few closely related species of arthropods, which constitute the major, if not exclusive, vectors. Most blood-feeding arthropods have a feeding preference for a few vertebrate species, determining the vertebrate species involved in maintaining virus transmission.

Many vectors of human viruses prefer nonhuman vertebrate blood sources for their perpetuation (ie, zoonotic viruses), including birds, rodents, or livestock (eg, Japanese encephalitis virus, tick-borne encephalitis, Rift Valley fever virus). In these systems, humans are infected when the vector "accidently" feeds on humans as an alternate blood meal, and the magnitude of circulating virus among the primary host and vector is a determinant of human risk of infection. However, humans are the major vertebrate host capable of maintaining cycles of other vector-borne viruses, such as dengue virus. In urban areas, humans are the primary reservoir maintaining transmission of some vector-borne viruses such as yellow fever, Zika, and chikungunya viruses. In these systems, transmission and human infection rates differ greatly by setting (ie, urban vs sylvatic vs intermediate).[32] Examples of transmission and maintenance patterns are listed in Table 13.4.

For some vector-borne viruses, there are alternative maintenance cycles limited to the arthropod host, with transovarial or sexual transmission from generation to generation of the arthropod host. These arthropod cycles may be critical in the perpetuation of the virus during adverse conditions, such as overwintering, but otherwise play a minor role in viral dissemination.

Terminal Hosts

A *dead end* or *terminal host* is one not involved in maintaining viral transmission. In these systems, humans are occasional terminal hosts for viruses maintained in animal cycles. These viruses use the range of transmission modes, including vector-borne (eg, Japanese encephalitis virus, West Nile virus), airborne (eg, Hantaviruses), and direct contact (eg, rabies virus). For example, maintenance of the West Nile transmission cycle is primarily within birds. Infected humans do not develop sufficiently high levels of viremia to transmit to mosquitoes and are, therefore, terminal hosts. Rabies virus is maintained in bats and other wildlife and domesticated dogs but can be transmitted to humans most often by dog bite contact. Of course, there are many examples of emerging zoonotic viruses, which initially spread from animals to humans in the recent past and then sustained human to human transmission (ie, HIV, SARS-CoV-2; see section below on viral emergence).

Endemic and Epidemic Patterns of Transmission

Endemic transmission refers to recurring or relatively stable transmission of a virus within a population. Epidemic transmission, on the other hand, refers to the rapid spread of a virus in which incidence exceeds a baseline or expected number of new infections. Less infectious viruses with long generation times tend to have endemic patterns with consistent infection rates over time with little seasonal fluctuation. Examples include sexually transmitted viruses such as HIV. An estimated 40 000 incident HIV infections have occurred each year in the United States over the past decade.[8] Some viruses exhibit seasonal endemic patterns of transmission,[56] that is, seasonal changes in the number of infections within a year but consistent annual number of infections. Seasonal variation in transmission can be driven by host behaviors, immunity, or environmental conditions impacting vector abundance.[34] Examples include viruses that do not induce protective immunity to reinfection, such as dengue virus, rotavirus, or influenza viruses. Viruses that are highly infectious with a short time interval between successive cases will quickly infect a susceptible population. If the viral infection also confers long-term protection against reinfection, we can expect persistent fluctuations or epidemic cycles as a result of the buildup and then depletion of a susceptible individuals born into the population.[29] Examples include many childhood infections such as measles, rubella, or varicella viruses. The duration of the interepidemic period depends in part on the number of susceptible individuals.[4] Human population sizes and birth rates play a critical role in determining the rate of new susceptible individuals, but, for vaccine-preventable viral diseases and settings with high vaccine coverage, vaccination effectively reduces the size of the susceptible population and lengthens interepidemic periods.

Within Host Persistence

Viral diseases can be broadly categorized by their persistence within the host as acute or chronic. Acute infections can be further characterized by the duration of immunity into those that result in long-term protective immunity and those that result in only short-term immune protection. Infections that do not confer long-term protection against reinfection are more common and result from multiple different mechanisms, including rapid viral evolution, cocirculating strains, or variable gene expression. For example, rapid evolution of influenza virus results in short-term immunity to prior strains. High mutation rates of genes encoding surface proteins leads to antigenic drift and viral reassortment during coinfections leads to antigenic shift. Viral infections that result in life-long sterilizing immunity are less common and include infections with measles, rubella, and smallpox viruses. It is not completely understood how and why some viral infections induce long-term protective immunity.

Whether a viral infection is persistent or acute and immunizing or nonimmunizing informs expectations for age-specific incidence and prevalence. Acute viral infections that induce long-term protective immunity mostly occur in infants and young children as most older individuals acquire infection and become immune. As a result, we often call these “childhood” infections even though susceptible adults can become infected. Acute viral infections that do not induce long-term protective immunity, depending on the duration of protection, often have attack rates that are similar across ages, resulting in age-invariant incidence and prevalence. In contrast, the prevalence of persistent viral infections increases with age.

Quantifying Transmission and the Basic Reproductive Ratio

The transmissibility of viral infections may be quantified by the basic reproductive ratio (R_0). R_0 is defined as the average number of secondary infections initiated by a single infectious individual in a completely susceptible population.[45] The basic reproductive ratio is a central concept in infectious disease dynamics. For immunizing infectious diseases in randomly mixing host populations, R_0 can be used to inform (1) the threshold for which the incidence of a viral infection can increase in a population ($R_0 > 1$); (2) the critical threshold for vaccine-induced herd immunity ($p_c = 1-1/ R_0$); (3) the expected final epidemic size in the absence of new susceptible individuals; and (4) the mean age of infection given a specified birth rate.[2] R_0 is determined by the transmissibility of the virus, contact rate between susceptible and infected individuals, and the duration of infectiousness. Examples of R_0 are 12-18 for measles virus, 5-7 for polioviruses and rubella virus, and 1.5-2.0 for influenza viruses, although there is a wide range depending on the setting.[2]

A more realistic measure to quantify the transmissibility of viral infection is the effective (or net) reproductive number (R). R is defined as the average number of secondary infections initiated by a single infectious individual in a partially immune population because of previous virus exposure or vaccination. R is smaller than R_0 because not all individuals in the population are susceptible. When R is >1, the incidence of infection increases; when R is <1, the incidence wanes. When R equals 1, the number of infections is constant. Control programs aim to reduce R to below 1 to achieve disease elimination.

A simple mathematical expression that captures the key parameters for a directly transmitted virus is $R = \beta cXD$, where β represents the transmission coefficient (ie, the probability of infection given contact between a susceptible and infected individual), c is the average number of contacts between susceptible and infectious individuals per unit time, X is the proportion of susceptible individuals, and D is the duration of infectiousness. As can be seen from this simple equation, control of a viral infection could be achieved by reducing the probability of infection

given effective contact (eg, masking), reducing effective contacts (eg, isolation and quarantine), reducing the proportion of susceptible individuals (eg, through vaccination), or reducing the infectious period (eg, treatments to reduce viral load and thus contagiousness).

Modeling Viral Dynamics

Mathematical models of viral dynamics are widely used by infectious disease epidemiologists to understand temporal and spatial patterns of virus transmission and persistence within populations, and to evaluate the potential impact of strategies to control or eliminate transmission. Much of the theory of viral dynamics and disease control has been developed using mathematical models, specifically mechanistic models (eg, the utility of R_0 and R as described above). Mechanistic models differ from statistical models used in other branches of epidemiology because their structure includes proposed mathematical relationships capturing biological mechanisms that drive infection dynamics.

The use of mechanistic models in infectious disease epidemiology has a long history. One of the earliest was a mathematic model of malaria transmission developed by Sir Ronald Ross in the early 20th century. In these early works, Ross laid out a mechanistic description of contagion and evaluated a threshold of mosquito populations below which malaria transmission could not be maintained. Subsequent models of infectious disease dynamics were developed by Wade Hampton Frost and Lowell Reed (the Reed-Frost model), in which the incidence of newly infected persons was based on the probability of contact between susceptible and infectious hosts.[1] The models developed by W. O. Kermack and A. G. McKendrick were the first to divide the population into the three classes of individuals discussed earlier (susceptibles, infectious, and recovered; hence, SIR models) and estimated the number of new infections as a function of the number of susceptible and infectious individuals.[48] These deterministic, compartmental models (based on difference or differential equations depending on whether time is treated as a discrete or continuous variable) were expanded and popularized by Roy Anderson and Robert May[2] and became the basis for much infectious disease modeling. Variations on the basic SIR model include the addition of other compartments or classes of individuals (eg, individuals infected but not infectious and infants protected by maternally acquired immunity), age- and space-specific contact patterns, and stochastic processes.

Mechanistic models can differ by scale, approach, and level of detail but are powerful tools that rely on implicit and explicit assumptions through which interpretation of model output is critical. Useful models balance the goals of the research question and simplicity. Models can provide a conceptual framework for viral transmission dynamics, identify which variables are most important for transmission and require careful measurement, and generate new hypotheses. Models can be used to estimate the likely size of an epidemic, its time course and periodicity, the reproductive number R, the expected impact of various interventions, evaluate strategies for control, and forecast disease burden. More complex models require additional underlying assumptions and parameter estimates and can be less intuitive. Model validation against epidemiologic data is a critical component of model building, use, and interpretation.

Measles is a useful system for infectious disease modelers to showcase dynamic mechanisms of transmission and control because it is a fully immunizing infection that is highly infectious and has a long time-series available because of the readily distinguishable clinical characteristics. Mathematical models of measles virus transmission allow for exploration of the impact of various vaccination strategies, including the optimum age at measles vaccination and the use of mass vaccination campaigns that would otherwise be difficult to evaluate in epidemiologic studies.[19] Model results suggest that the best age at which to vaccinate against measles depends critically on the age distribution of cases of infection prior to the introduction of control measures.[57] Introduction of mass measles vaccination induces a temporary phase of low incidence of infection before the system settles to a new pattern of recurrent epidemics,[58] and frequent, relatively low coverage mass vaccination campaigns are effective in reducing measles virus transmission.[51]

Phylodynamics

Infectious disease epidemiologists are increasingly interested in linking immunological, epidemiological, and evolutionary processes to understand how viruses are transmitted and evolve, a field referred to as phylodynamics.[39] Because of the high mutation rates of viral pathogens, evolutionary and epidemiologic processes take place on a similar time scale.[76] According to this framework, phylodynamic processes that determine the degree of viral diversity are a function of host immune selective pressures and epidemiologic patterns of transmission.

Since the term phylodynamics was introduced in 2004, the field has expanded dramatically, enabled by the decreasing cost of genetic sequencing and increasing availability of viral sequence data[70] Open-source software to analyze viral sequence data (eg, BEAST[22]) has contributed to the expansion of available data, allowing scientists to readily apply nonlinear evolutionary models. Sequence data are used to understand details of virus evolution and transmission, including the frequency and source of virus introductions, or deconstructing the role of “hotspots” to maintain spread.[33,88] The knowledge gleaned can be leveraged to anticipate future spread and develop control strategies. The utility of phylodynamic analysis is also applicable to clinical settings, including linking closely related cases to predict phenotypes such as drug resistance and virulence.[70]

The Zika virus epidemic in the Americas began in May 2015 with the first reported case in Brazil. Zika virus was subsequently reported from many countries throughout Central America, South America, and the Caribbean.[49] Genomic sequencing combined with phylogenetic, epidemiologic, and environmental data were used to understand Zika virus transmission over space and time. These analyses tracked Zika virus transmission across national boundaries and assessed the extent of undetected spread throughout the region.[88] Phylodynamic analyses also informed understanding of biannual peaks of viral transmission, likely driven by environmental conditions that impact the vector *Aedes* sp. mosquitoes, despite the other modes of transmission (eg, blood transfusion, sexual contact, vertical transmission) that were not readily impacted by environmental factors.[88]

Influenza A viruses display endemic seasonal patterns of transmission with annual outbreaks occurring in during “winter” months in temperate climates (November to March in the northern hemisphere and from May to September in the southern hemisphere).[46] As a result of antigenic drift, a large pool of susceptible individuals at risk of infection is present every year. Phylogenetic analysis provided key evidence to inform the underlying causes of seasonal influenza A virus transmission. Rather than the virus remaining local within a host population between seasonal outbreaks, as previously proposed, global migration of infected individuals spreads the virus and contributes to seasonal emergence of influenza A viruses.[71] Countries in the tropics with persistent influenza virus reservoirs can serve as “sources” for “sink” countries in temperate zones.[78]

Viral Emergence

One of the most dramatic aspects of infectious disease epidemiology is the appearance of a new viral disease, as the world is well aware following the emergence of SARS-CoV-2 and the resulting pandemic.[74] This may reflect the true appearance of a new virus in the population; an increase in the case infection ratio so that an endemic infection is associated with a marked increase in disease incidence; or lastly, the recognition of an existing but previously unidentified disease that can now be clearly diagnosed owing to new laboratory tests accompanied by heightened practitioner awareness.

New viral diseases may be emerging as zoonoses at an increasing frequency, with SARS-CoV-2 being the most recent and dramatic example.[93] Several secular trends have enhanced the probability of emergence of new viral diseases (Fig. 13.6).[90] First, the population of the world has inexorably continued to increase, and urbanization has concentrated a higher proportion of people in densely populated areas, facilitating the transmission of any new virus. Second, modern transportation permits the carriage of infections around the globe within a single incubation period. Third, man-made perturbations of the environment are occurring at an increasing pace, thus increasing the likelihood of transmission of zoonotic and arboviral infections to humans. Finally, advances in fundamental virology have made available a new armamentarium of methods for the detection of previously unknown pathogenic viruses. All these factors were in play with the emergence and spread of SARS-CoV-2.

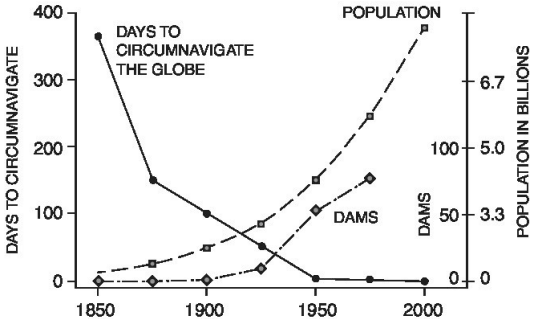

FIGURE 13.6 **Since the middle of the 19th century, many global changes have enhanced the probability of the emergence of new viral diseases of humans and animals**. This chart depicts three examples of such trends. The growth in the human population has been accompanied by a massive increase in large urban populations, markedly increasing the risk of epidemic spread of infection. Dramatic reductions in the time to travel over long distances have increased the possibility of global transport of infectious agents over short periods of time. The number of large dams (dams >75 meters high built in the United States from 1890 to 1975) exemplifies increases in man-made manipulation of the environment. (Data from Anonymous. *Atlas of World Population History*. Penguin Books and Allen Lane; 1978; Anonymous. *Statistical Yearbook*. 38th Issue, 1990/1991. United Nations Publications; 1993; Mandzhavidze NF, Mamradze GP. *The High Dams of the World*. Academy of the Sciences of the Georgian SSR and Clearinghouse for Federal Scientific and Technical Information; 1994.)

Epidemics

Causes of Epidemics

Epidemics may result from an increase in viral virulence, an increase in the number or proportion of susceptible individuals exposed to the virus, an increase in the number or proportion of susceptible individuals in a population, recent emergence of a new virus, or re-emergence of an existing virus. Expanding knowledge of basic virology provides molecular insights into the determinants of viral epidemics. For example, generation time and transmissibility of a virus, that in turn impact the magnitude and timing of epidemics, are influenced by specific viral genes. Mutations in the gene encoding the spike protein of SARS-CoV-2 resulted in increased transmissibility and shortening of the generation time.[53] In certain instances, it is possible to identify some of the molecular determinants of each of these four mechanisms that are associated with epidemics.

Increase in Viral Virulence

In many instances, an outbreak is clearly associated with a virus strain of high virulence that increases the ratio of reported cases to infections. Such occurrences are probably quite frequent but are rarely well documented. Theoretical models inform our understanding of the selection for viral virulence at the onset of an epidemic, despite selection for less virulent or latent virus later in the epidemic as the susceptible population decreases in size.[3] Most viruses exhibit great natural variation in virulence. One classical example is variola virus, which caused virulent forms of smallpox (variola major) in India and Africa and much milder disease (alastrim or variola minor) in South America (Table 13.3). It was known for many years that natural isolates of poliovirus varied greatly in their neurovirulence, and attenuated isolates were the starting point for the development of avirulent vaccine strains. Molecular analysis identified the genomic determinants of virulence, which are located both in the structural genes and in the noncoding 5′ region of the genome.[67] This natural variation might have been a factor in the severity of poliomyelitis epidemics of the past. SARS-CoV-2 variants have also differed in virulence as well as transmissibility.[41]

Increase in Susceptible Population Exposed to the Virus

An enhanced mode of transmission or a change in factors that increase host exposure can increase the susceptible population exposed to the virus. Ecological factors, in particular climatic conditions (rainfall, temperature, etc.), that maximize the vector population can increase human risk of vector-borne virus infection. Additionally, a change in the interplay between environment and human behavior can increase human exposures to sylvatic disease cycles. Examples include population growth and spatial expansions of farming and agriculture increasing interactions between wildlife, vectors, livestock, and humans.[47]

Increase in Population Susceptibility

Waning immunity or viral variants that escape prior immunity can result in increases in population susceptibility. Susceptible individuals also

increase in a population through births and migration and when transmission temporarily ceases. Viruses that are highly infectious with immunizing infections and short generation times will quickly be transmitted in a susceptible population resulting in fluctuations or epidemic cycles.[29] Measles incidence in Iceland over a 50-year period is shown in Figure 13.7, during which time epidemics alternated with periods with no measles virus transmission. When measles virus invades an isolated island population, the virus spreads widely and quickly and fades out when the density of susceptible individuals drops below the level needed to sustain virus transmission. The virus disappears entirely, allowing susceptible individuals to accumulate as new cohorts of infants are born. Once enough susceptible individuals have accumulated, a single importation of measles virus can initiate a new epidemic.[84]

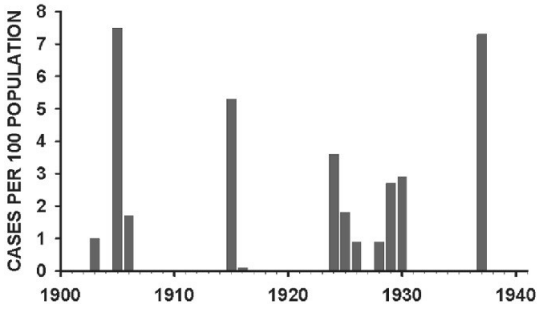

FIGURE 13.7 **Measles incidence in Iceland from 1900 to 1940**. (Data from Tauxe R. Measles incidence in Iceland, 1900-1940. Unpublished report. 1979.)

Emergent or Re-emergent Viruses

SARS-CoV-2 is the most well-known and recent example of a new virus that led to an epidemic (more specifically, a global epidemic or pandemic). Emergent viruses infect and transmit among a completely susceptible population allowing for high attack rates depending on the infectiousness of the virus. A time series of global cases reported to the World Health Organization show the epidemic curve in Figure 13.8 during which different strains of the virus resulted in new waves of cases. SARS-CoV-2 is an epidemic virus likely to become endemic if reinfection is common.[83]

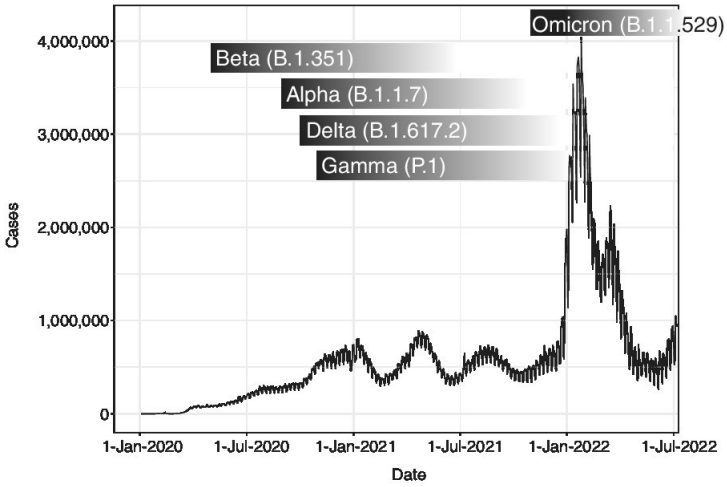

FIGURE 13.8 **Epidemic curve of global SARS-CoV-2 cases reported to the World Health Organization**. Temporal changes in variants of concern are shown with the starting date based on the earliest documented sample. (Data were extracted from World Health Organization website on 11 July 2022, https://covid19.who.int/data, aggregated across countries by day, and plotted as a time series & https://www.who.int/activities/tracking-SARS-CoV-2-variants.)

While seasonal influenza is considered an endemic disease across the world, the segmented genome of influenza viruses enables exchange of entire gene segments when cells are coinfected with two influenza viruses. Reassortment can lead to a major shift in the antigenicity of the virus if the new virus bears a hemagglutinin that is novel for the human population. Reassortment between an avian influenza virus and the previously circulating human influenza virus occurred in 1957 and 1968, with the appearance of the H2N2 and H3N2 pandemic influenza viruses, respectively. Animal influenza viruses also reassort in nature; the 2009 pandemic was caused a complex, triple-reassortant H1N1 swine influenza virus.[72] Such a radical shift in antigenicity provides the newly created virus with a global population that is relatively susceptible and, as a result, an influenza pandemic may occur.

Epidemic Investigation

The primary goal of an epidemic or outbreak investigation is to control the spread of disease. This requires identifying the etiologic agent (including but not limited to viruses), its source, and the mode of transmission. During an outbreak investigation, epidemics can be classified as either point source, common source, or propagated. An epidemic curve (ie, time series of cases) is a useful outbreak investigation tool for delineating these modes (Fig. 13.9).

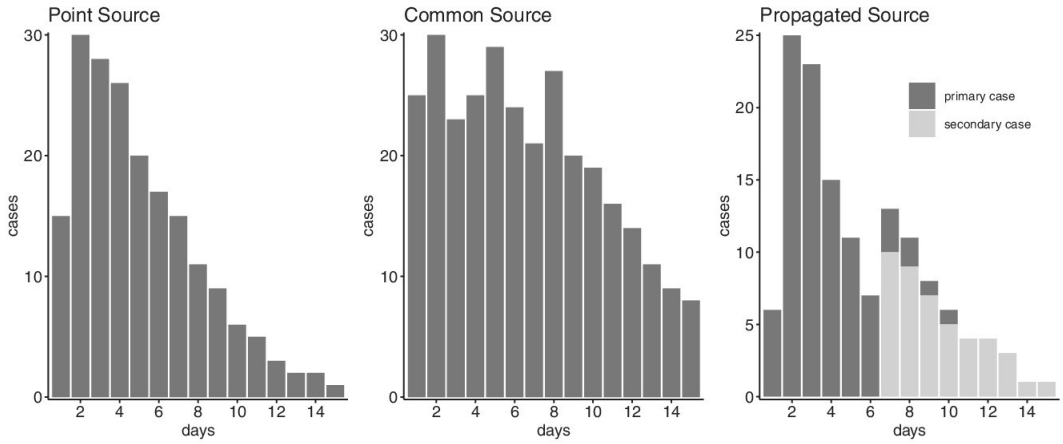

FIGURE 13.9 Examples of epidemic curves showing how the shape of the curves provides insight into the modes of transmission and duration of the incubation period. The three epidemic curves represent a point source (**left panel**), common source (**center panel**) and propagated source (**right panel**).

Point Source Outbreak

Point source outbreaks are those in which a susceptible population is exposed at a discrete point in time. As the name implies, pathogen exposure occurs from a point source, often food, water, or aerosol. Point source outbreaks are explosive because of the simultaneous exposure. The epidemic curve is characterized by the rapid rise and only slightly slower fall in the number of cases, with variation based on individual heterogeneity in incubation periods and clinical phases. The most common point source outbreaks in the United States are those involving contaminated food.[86]

Common Source Epidemic

Common source epidemics are the result of exposure to a pathogen from a common source, including food, water, aerosol, or injected product. But unlike a point source outbreak, a common source epidemic continues to expose individuals to the pathogen. The epidemic curve for a common source outbreak includes a sudden rise in cases but instead of dropping quickly, cases continue as individuals continue to be exposed.

Propagated Epidemic

Propagated epidemics involve host to host spread of the pathogen. A propagated epidemic typically includes an increase in the number of cases following exposure. As the number of susceptible individuals from the initial exposure decreases, so will the number of cases. Secondary cases resulting from person to person transmission result in a secondary rise in the number of cases one incubation period after the initial peak in cases. Propagated epidemics result in the largest number of epidemic cases owing to the perpetuation of transmission. Finally, some epidemics are neither point-source, common-source, nor propagated from person to person, such as epidemics of zoonotic or vector-borne diseases.

Perpetuation and Eradication of Viruses

All viruses, whether they cause acute or chronic infections, are capable of persisting in populations because perpetuation is a requirement for survival. Eradication is the converse of perpetuation and represents the ultimate method for control of an infectious disease. To determine the potential for eradication, it is necessary first to understand the requirements for perpetuation.

The principal ecological patterns of viruses in host populations have been discussed previously, with emphasis on human viruses. In most instances, a virus is perpetuated in a single species, and discussion will be limited to this dominant ecological pattern. Parameters that determine perpetuation include characteristics of the human population and the virus. Population determinants include the size of the population, the rate at which new susceptible individuals are introduced (largely through births), the density of the population, and the proportion of the population that are susceptible. Viral determinants include transmissibility, generation time, and duration of infectiousness (acute or persistent). These three viral variables determine the rate of spread of a virus through a population for a given set of population parameters. Paradoxically, an agent that spreads rapidly may exhaust susceptibles and disappear more quickly from a small population than a virus that moves indolently through the same population. Perpetuation will be discussed separately for large and small populations, because population size is such an important determinant.

Small Populations

For small populations, a crude prediction of sustained transmission may be made by determining the number of susceptible individuals entering the population per generation period. If this number is less than one, persistence will almost certainly not occur.[95] Small primitive human populations that have minimal contact with the outside world are occasionally available for serological study. These populations provide a unique opportunity to determine the ability of viruses to perpetuate in human hosts. There are two principal patterns.[5] Viruses that cause acute short-term infections produce abrupt outbreaks after introduction into human populations and then die out until reintroduced. Viruses that are capable of persisting in individual hosts as chronic infections can sustain transmission in small, isolated groups.

Animal populations differ radically from human populations in their relatively rapid rate of turnover—a critical variable in sustained virus transmission. Mean life expectancy in many animal populations ranges from 6 to 12 months, in contrast to a range of 30-70 years for humans. However, the kinetics of infection is often longer in an individual human than animal host. For instance, the median incubation period (to rash)

is about 14 days in smallpox and 10 days in mousepox. The consequence is that sustained transmission may occur in animal populations with rapid turnover that are much smaller than the minimum human population required to sustain a similar virus.

Laboratory animal colonies have provided an opportunity to document the quantitative impact of population turnover. Perpetuation in such colonies is of more than academic interest because enzootic infections present an important practical problem that frequently confounds biomedical research. One documented example is ectromelia (mousepox) that closely resembles variola in humans.[23] Infection is initiated either by entry through the skin or by inhalation and results in a generalized poxlike rash of the skin from which aerosolized virus is transmitted. As in smallpox, infections can be fatal, although recovered mice are solidly immune. Ectromelia is a relatively infectious agent and a notorious cause of acute and devastating epizootics. When a few infected mice are placed in a larger group of susceptible animals, the virus spreads rapidly, all mice are infected within two incubation periods, and many mice die. In these circumstances, ectromelia would not be expected to perpetuate in a small mouse population. However, a series of classical studies demonstrated that this virus was readily perpetuated in a population of 100-200 animals because of the more rapid population turnover in mice.[36]

Large Populations

To follow the pattern of viral infection in large populations, it is generally necessary to rely on reportable diseases that can be identified by clinical observation and that exhibit relatively high case infection ratios. Measles is one of the few viral diseases that meets these criteria; however, even measles surveillance has its limitations, considering only a fraction (usually 10%-50%) of cases are reported. Table 13.6 shows that in cities of 200 000-300 000, measles faded out for at least 1 month in 56 of the 100 years recorded. Because a population of 300 000 might be expected to experience about 5000 cases of measles in an average year, it is initially surprising that this would be insufficient to maintain measles virus transmission. The explanation becomes more apparent when the seasonal cycle of measles is examined.[95] Data for the city of Baltimore (population 900 000) for 32 years of observation showed that annual reports averaged about 5000 cases, representing about 35% of cases; incidence rates ranged more than 100-fold from the peak in March to the seasonal low in August, when about 10 cases were reported. Because there were about three generation periods per month, this represents no more than 3 cases per generation period, which is barely sufficient to perpetuate the virus. A similar city of 300 000 would experience only 1 case per generation period at the seasonal trough; in such an instance, it is quite plausible that periodic fadeouts would occur.

TABLE 13.6 Fadeouts of measles in North American cities, 1921-1940[a]

Size of City (Millions)	Number of Cities	Average Annual Measles Reports (Thousands)	Years of Study	Years With Fadeout
1.0-7.5	6	3.5-21	120	0
0.5-0.9	9	2.8-4.0	180	1
0.2-0.3	5	0.9-2.1	100	56

[a]Estimate of reporting ranged from 14% to 55% of measles incidence. *Large cities:* New York, Chicago, Philadelphia, Detroit, Los Angeles, Montreal; *medium cities:* Cleveland, Baltimore, Boston, Toronto, Washington, Pittsburgh, Milwaukee, Buffalo, Minneapolis; *small cities:* Vancouver, Rochester, Dallas, Akron, Winnipeg.

Data from Bartlett MS. The critical community size for measles in the United States. *J R Stat Soc A*. 1960;123:37-44; Yorke JA, London WP. Recurrent outbreaks of measles, chickenpox, and mumps. II. Systematic differences in contact rates and stochastic effects. *Am J Epidemiol*. 1973;98:469-482.

This analysis suggests that persistence of a short-cycle infection in a large population depends on the relationship between incidence during the seasonal trough and the generation time. Influenza virus, with a generation time of 1-3 days, is maintained in human populations through the seasonal trough by shuttling between Northern and Southern hemispheres each year.

Requirements for Eradication

Elimination refers to the interruption of virus transmission in a defined geographic area (eg, country or region), and eradication refers to the global interruption of virus transmission. The history of smallpox, and more recently rinderpest virus, demonstrates that eradication is an attainable objective for selected human and animal viral infections. The salient epidemiologic features that made smallpox eradication possible were its relatively long incubation period (about 14 days) and low infectiousness, the near equivalent durations of the latent and incubation periods, its marked seasonality, a case to infection ratio approaching 1, and the lack of a nonhuman reservoir. These features made it possible to abandon mass vaccination in favor of a search and containment strategy in which local outbreaks were identified and aborted by intensive immunization around each focus.[31] The paucity of local outbreaks during the seasonal trough were essential to the success of this strategy.

Eradication of Polioviruses

Two poliovirus vaccines (inactivated poliovirus vaccine [IPV] and oral poliovirus vaccine [OPV]) were introduced in the period from 1955 to 1963 in the United States, and their application led to the elimination of circulating wild polioviruses around 1970.[68] Wild polioviruses were eliminated from the Western Hemisphere by 1990 as a result of immunization programs in Latin America, and the World Health Organization set a goal of global eradication in 1988. Worldwide efforts reduced polio cases from an estimated 350 000 in 1988 to fewer than 1000 in 2000. However, since then, elimination efforts have stalled owing to the persistence of wild polioviruses in Afghanistan and Pakistan. In 2021, a case of paralytic poliomyelitis due to wild poliovirus type 1 was reported in Malawi 1 year after the World Health Organization African Region was certified as free from wild-type polioviruses,[92] a further setback to the Global Polio Eradication Initiative. There have been two major impediments to eradication, first the failure to immunize a sufficiently high proportion of infants and young children in settings where transmission of enteric infections is very intense and, second, a low “take” rate of OPV, owing in part to competition between the three serotypes in the vaccine. However, OPV presents another complicating issue, namely the tendency to revert to a more virulent phenotype on human enteric passage. Vaccine-derived polioviruses have caused outbreaks of poliomyelitis in settings where weak immunization programs have left many children susceptible and capable of spreading OPV shed by those who were immunized. Circulating vaccine-derived poliovirus (cVDPV)

cases were reported from 31 countries during 2020-2021, further hindering polio eradication efforts.[92]

Eradication of Measles Virus

The feasibility of measles eradication has been debated for many years. Measles meets criteria for a disease that can be eradicated: (1) there is no animal or environmental reservoir, and humans are critical to maintaining transmission; (2) accurate diagnostic tests are available; (3) measles vaccine and existing vaccination strategies are effective and safe; and (4) measles transmission has been interrupted in a large geographic area (eg, nationwide) for a prolonged period. The WHO conducted a comprehensive assessment of the feasibility of measles and rubella eradication in response to a request from the World Health Assembly.[61] The report concluded that a time-bound measles and rubella eradication goal should only be set when substantial and measurable progress has been made, and the strategies, resources, and commitment are likely to be in place to interrupt the final transmission pathways.

Several potential biological obstacles to measles eradication should be considered.[62] Persistent infection with transmissible measles virus would pose a biological barrier to eradication. Measles virus is known to establish persistent infection in persons with subacute sclerosing panencephalitis (SSPE); however, virion assembly and budding is defective and multiple mutations occur throughout the measles virus genome.[40] As a consequence, infectious measles virus is not present. Theoretically, selective pressure on measles viruses to mutate neutralizing epitopes and escape protective immune responses induced by vaccines could be a biological obstacle to measles eradication. However, despite the high degree of genetic variation expected of an RNA virus, mutations in the measles virus genome have not reduced the protective immunity induced by measles vaccines.[64] Subclinical infection resulting in sustained measles virus transmission also could pose a barrier to eradication.[63] However, sustained measles virus transmission among partially immune individuals without clinical disease would be highly unlikely.

Eradication of Rinderpest Virus

Rinderpest virus, a pathogen of cattle and many other domestic and wild artiodactyl species, was declared eradicated on May 25, 2011, following widespread vaccination and control efforts coordinated by the Global Rinderpest Eradication Programme.[59] Rinderpest virus is a *Morbillivirus* closely related to measles virus, transmitted by close contact with an infected animal via inhalation of virus-containing nasal, oral, or fecal secretions. Infection resulted in severe diarrhea leading to dehydration and death. Rinderpest impacted human health in Europe and Africa by decimating cattle populations, leading to famine, disease, and death. What is remarkable about rinderpest eradication is that the virus has many potential reservoirs, both domestic and wild, and yet the goal of eradication was achieved through widespread vaccination of cattle and buffalo, establishment of effective surveillance programs, and regional coordination of control efforts.

Applications of Epidemiology

Outbreak Investigation to Identify Etiological Agents

Epidemiology grew out of outbreak investigations of infectious diseases. Early outbreak investigations, such as investigations of cholera by John Snow in the mid-19th century, were conducted before the widespread acceptance of germ theory but nonetheless comprised hypothesis-driven research. The steps of an outbreak investigation through which hypotheses are formed generally consist of defining a case, assessing the distribution of cases over time to inform the mode of transmission (including tracking subsequent cases), characterizing potential risk factors, and investigating possible sources by exploring common exposures among cases. Epidemiologically, it may be difficult to infer whether the cause is a virus or other infectious agent. Epidemiologic observations, however, can suggest the mode of transmission and the period of infectiousness, which are important for collection of specimens for virus isolation.

Viruses that cause more severe disease or death are more readily identified due to their readily noticeable clinical presentation. Hantavirus pulmonary syndrome (HPS) was unknown in 1993 when first identified but later was discovered to be caused by different hantaviruses. The investigation was triggered by an illness characterized by respiratory distress and death in two otherwise healthy, young individuals residing in the same household in New Mexico, USA.[10] As part of the investigation, epidemiologists identified other individuals who died of similar acute respiratory failure, but laboratory tests failed to identify an etiologic agent. Virologists conducted thorough testing of tissue samples from patients with similar clinical histories and were able to identify a previously unknown hantavirus.[11] Throughout the remainder of 1993, investigators trapped as many different species of rodents living near the households and places of occupation of the cases and finally identified the viral reservoir in deer mice.[15] The last step was to investigate the connection between deer mice and cases. A case-control study design was used (given the low incidence of disease) among surviving cases or people and controls matched by geographic location. Cases had more rodents around living near their houses and were more likely to clean around the house and plant in outdoor gardens.[96] National surveillance for HPS began 1995 and 816 cases were reported in the United States through 2019, as seen in Figure 13.10. Most cases (94%) occur in states west of the Mississippi River in states more prone to drought.

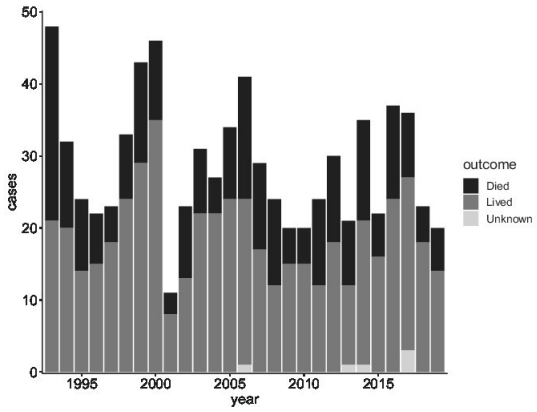

FIGURE 13.10 **Case count of hantavirus disease reported 1993 to 2019 in the United States**. Data were reported to US CDC's Viral Special Pathogens Branch and Collected by the Nationally Notifiable Disease Surveillance System. Annual case count is grouped by the outcome of the case (lived, died, unknown). (Source US CDC. *Reported Cases of Hantavirus Disease*. Accessed April 20, 2022. https://www.cdc.gov/hantavirus/surveillance/index.html)

Epidemiologic observations may also contribute insights into more obscure persistent infections associated with atypical chronic diseases. The spongiform encephalopathies or prion diseases are an example. This group of agents includes scrapie of sheep, kuru and Creutzfeldt-Jakob disease of humans, and bovine spongiform encephalopathy.[17] The initial definitive evidence that scrapie, a chronic degenerative neurological disease, could be transmitted by an infectious agent and occurred as an unwanted complication of the use of louping ill vaccine was made in Scotland in 1935.[37] Table 13.7 shows that three lots of vaccine against louping ill, a flavivirus, were injected into sheep in Scotland. Unexpectedly, there was a very high incidence of scrapie in recipients of one of the three lots. Subsequent epidemiologic investigation showed that louping ill vaccine was prepared by formalin treatment of virus grown in sheep brain and that several of the sheep used were probably incubating scrapie because their parents subsequently came down with the disease. Prions are extremely resistant to inactivation by formalin; thus, contamination of the vaccine was readily explained. Prior to this episode, it was not generally accepted that scrapie was attributable to a transmissible agent with an incubation period of several years.

TABLE 13.7 Occurrence of scrapie in sheep immunized with louping ill vaccine, Scotland, 1935[a]

Vaccine Batch	Number of Sheep Vaccinated	Percentage With Scrapie
1	>22 000	<0.1%
2	18 000	5%
3	>4000	<0.1%

[a]Batch 2 was prepared from 122 sheep infected with louping ill; of these, 8 were offspring of scrapie-affected ewes. Interval between subcutaneous injection of vaccine and onset was 20 months or longer.

Data from Greig JR. Scrapie in sheep. *J Comp Pathol*. 1950;60(4):263-266.

Evaluation of Vaccine Efficacy and Safety

The ultimate test of viral vaccines is their efficacy and safety in humans. Epidemiology provides well-established methods for the design and analysis of prospective studies of vaccine efficacy and effectiveness.[43] Vaccine efficacy estimates come from prelicensure, randomized, double-blind phase three clinical trials, evaluating the direct effect of vaccination on the individual (including individual susceptibility to infection, disease, death, or infectiousness). Vaccine effectiveness estimates, which measure direct protection to the vaccinated as well the indirect effect on the population, come from postlicensure observational studies. These are illustrated by the 1954 field trial of poliomyelitis vaccine.[55] In this trial, a large number of children in the first three grades of elementary school were enrolled. As shown in Table 13.2, the trial comprised two different study designs—namely, placebo and observed. In the placebo design, children fell into three categories: vaccinated, placebo inoculated, and nonvolunteers (uninoculated). In the observational study, the three groups were vaccinated second graders, nonvolunteer second graders (unvaccinated), and controls (all children in the first and third grades). The most valid comparison was between vaccinated and placebo-inoculated children in the placebo areas. This group yielded an estimated efficacy of 72% for paralytic poliomyelitis, whereas the observed control area yielded an efficacy of 63%. The discrepancy was explained by the fact that the nonvolunteer children in both areas experienced an attack rate that was considerably lower than that of the placebo controls or the observed controls. Apparently, volunteer children were at higher risk (presumably because they included a higher percentage of susceptibles) than were nonvolunteer children. Thus, the strict placebo control was the best comparison group. When the placebo study was further refined by limiting the analysis to laboratory-confirmed cases, the estimated efficacy was even higher (85%), underlining the importance of etiology-proven cases in vaccine evaluation. As described above, test negative designs can be a more efficient approach to estimate vaccine effectiveness while reducing selection biases.

Epidemiologic studies can also play a critical role in documenting adverse events associated with vaccines. Postlicensure monitoring of vaccine-related adverse events is conducted using passive surveillance systems, through which individuals, health care providers, and vaccine manufacturers can report suspected adverse events. These passive surveillance systems serve to generate hypotheses regarding potential causal relationships between vaccines and adverse outcomes and thus serve as early warning signals. More rigorous epidemiological studies then need to be conducted to establish causality.

The first rotavirus vaccine was licensed in 1998 (RotaShield), but the CDC recommended health care providers suspend use of the vaccine shortly thereafter in July 1999.[12] Between December 1998 and June 1999, ten cases of intussusception in infants were reported to the US passive surveillance systems for adverse vaccine-related events, and the US CDC conducted epidemiological studies to evaluate a potential causal relationship between the rotavirus vaccine and intussusception. A case-control study in 19 states and a retrospective cohort study in managed care organizations were conducted. In the retrospective cohort study, data on the cases of intussusception and the vaccination status of infants were available from participating managed care organizations. The incidence rate of intussusception among vaccinated and nonvaccinated infants were compared and showed an increased risk of intussusception after administration of the rotavirus vaccine (Table 13.8).[50] In the case-control study, cases of hospitalized infants for intussusception were identified via medical records and were matched by age and hospital of birth to nonintussusception controls. Seventeen and 13% of cases and controls received the rotavirus vaccine, respectively. The risk of intussusception was increased 3-14 days after the first dose of the rotavirus vaccine.[65] As a result of these finding, the vaccine was no longer recommended and production was stopped. However, there are now several licensed rotavirus vaccines used around with world, and two licensed vaccines in the United States, and with a much lower risk of intussusception.

TABLE 13.8 Incidence rate and relative risk of intussusception after rotavirus vaccination

Vaccine Batch	Vaccinated	Never Vaccinated
Number of children	56 253	407 024
Person-years	13 541	126 492
Cases	9	31
Incidence rate	66	25
Relative risk	2.7	1.0 (reference)

Reprinted with permission from Kramarz P, France EK, Destefano F, et al. Population-based study of rotavirus vaccination and intussusception. *Pediatr Infect Dis J*. 2001;20(4):410-416.

Development and Assessment of Control Measures

In addition to vaccines, other control measures are often useful against viral infections. Such approaches seek to reduce the risk of exposure and are usually suggested by epidemiologic studies of transmission. A recent example is the prevention of respiratory droplet and airborne transmission of SARS-CoV-2 through masking, social distancing, and improved ventilation.[16] Other historical examples include reduction in the risks associated with blood or blood products for viruses such as hepatitis B and hepatitis C, HIV, and human T-cell leukemia viruses I and II. Another classical example is the reduction of urban yellow fever and dengue through control of *Aedes aegypti* mosquito populations. The use of epidemiologic management of viral infection is illustrated by a study of Bolivian hemorrhagic fever—an arenavirus that was endemic in certain rural villages.[85] Ecological studies suggested that this virus was enzootic in one species of peridomestic house-dwelling mouse—*Calomys callosus*—and was transmitted by aerosolized fomites. Table 13.9 shows a study in which rodent control was initiated in one-half of a village and then extended to the other half. The dramatic effect of rodenticides confirmed the hypothesis of the virus reservoir and its transmission mechanism and provided a practical approach to control of a serious viral infection. Sophisticated mathematical models have been used to evaluate control measures that cannot be tested in epidemiologic studies, including containment of a potential bioterrorist use of smallpox virus[42] and containment of pandemic influenza virus through vaccination[94] and the use of prophylactic antivirals.[24]

TABLE 13.9 Control of Bolivian hemorrhagic fever by reduction of the rodent reservoir, San Joaquin, Bolivia, 1964[a]

Ten-Day Period Beginning	New Cases, East Sector	New Cases, West Sector
April 1	12	9
11	8	17
21	12	8
May 1	7	18
11	4	14
21	2	9
31	3	12
June 10	0	10
20	5	7
30	0	0
July 10	0	0

[a]Rodents were controlled by trapping and poisoning, beginning in the East sector on May 1 and in the West sector on June 15.

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In Memoriam of Neal Nathanson, for his contribution to the fields of epidemiology and virology and whose prior chapter we revised.

REFERENCES

1. Abbey H. An examination of the Reed-Frost theory of epidemics. Hum Biol. 1952;24:201-233.

2. Anderson MR. Infectious Diseases of Humans. Oxford University Press; 1991.

3. Berngruber TW, Froissart R, Choisy M, Gandon S. Evolution of virulence in emerging epidemics. PLoS Pathog. 2013;9:e1003209.

4. Bjornstad ON, Shea K, Krzywinski M, Altman N. The SEIRS model for infectious disease dynamics. Nat Methods. 2020;17:557-558.

5. Black FL. Infectious diseases in primitive societies. Science. 1975;187:515-518.

6. Bobrovitz N, Arora RK, Cao C, et al. Global seroprevalence of SARS-CoV-2 antibodies: a systematic review and meta-analysis. PLoS One. 2021;16:e0252617.

7. Bolotin S, Osman S, Hughes SL, et al. In elimination settings, measles antibodies wane following vaccination but not following infection—a systematic review and meta-analysis. J Infect Dis. 2022;226(7):1127-1139.

8. Bosh KA, Hall HI, Eastham L, Daskalakis DC, Mermin JH. Estimated annual number of HIV infections horizontal line United States, 1981-2019. MMWR Morb Mortal Wkly Rep. 2021;70:801-806.

9. Cauchemez S, Bhattarai A, Marchbanks TL, et al. Role of social networks in shaping disease transmission during a community outbreak of 2009 H1N1 pandemic influenza. Proc Natl Acad Sci U S A. 2011;108:2825-2830.

10. Centers for Disease Control and Prevention. Outbreak of acute illness—southwestern United States, 1993. MMWR Morb Mortal Wkly Rep. 1993;42:421-424.

11. Centers for Disease Control and Prevention. Update: outbreak of hantavirus infection—southwestern United States, 1993. MMWR Morb Mortal Wkly Rep. 1993;42:495-496.

12. Centers for Disease Control and Prevention. Suspension of rotavirus vaccine after reports of intussusception—United States, 1999. MMWR Morb Mortal Wkly Rep. 2004;53:786-789.

13. Cevik M, Baral SD. Networks of SARS-CoV-2 transmission. Science. 2021;373:162-163.

14. Chakraborty C, Sharma AR, Bhattacharya M, Lee SS. A detailed overview of immune escape, antibody escape, partial vaccine escape of SARS-CoV-2 and their emerging variants with escape mutations. Front Immunol. 2022;13:801522.

15. Childs JE, Ksiazek TG, Spiropoulou CF, et al. Serologic and genetic identification of *Peromyscus maniculatus* as the primary rodent reservoir for a new hantavirus in the southwestern United States. J Infect Dis. 1994;169:1271-1280.

16. Chu DK, Akl EA, Duda S, et al. Physical distancing, face masks, and eye protection to prevent person-to-person transmission of SARS-CoV-2 and COVID-19: a systematic review and meta-analysis. Lancet. 2020;395:1973-1987.

17. Collins SJ, Lawson VA, Masters CL. Transmissible spongiform encephalopathies. Lancet. 2004;363:51-61.

18. Cummings DA, Irizarry RA, Huang NE, et al. Travelling waves in the occurrence of dengue haemorrhagic fever in Thailand. Nature. 2004;427:344-347.

19. Cutts FT, Dansereau E, Ferrari MJ, et al. Using models to shape measles control and elimination strategies in low- and middle-income countries: a review of recent applications. Vaccine. 2020;38:979-992.

20. Danon L, Ford AP, House T, et al. Networks and the epidemiology of infectious disease. Interdiscip Perspect Infect Dis. 2011;2011:284909.

21. Dowell SF. Seasonal variation in host susceptibility and cycles of certain infectious diseases. Emerg Infect Dis. 2001;7:369-374.

22. Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol. 2007;7:214.

23. Fenner F. Mouse-pox; infectious ectromelia of mice; a review. J Immunol. 1949;63:341-373.

24. Ferguson NM, Cummings DA, Cauchemez S, et al. Strategies for containing an emerging influenza pandemic in Southeast Asia. Nature. 2005;437:209-214.

25. Ferrari MJ, Grais RF, Bharti N, et al. The dynamics of measles in sub-Saharan Africa. Nature. 2008;451:679-684.

26. Fine P, Eames K, Heymann DL. “Herd immunity”: a rough guide. Clin Infect Dis. 2011;52:911-916.

27. Fine PE. The interval between successive cases of an infectious disease. Am J Epidemiol. 2003;158:1039-1047.

28. Fine PE, Clarkson JA. Measles in England and Wales—I: an analysis of factors underlying seasonal patterns. Int J Epidemiol. 1982;11:5-14.

29. Finkenstadt BF, Bjornstad ON, Grenfell BT. A stochastic model for extinction and recurrence of epidemics: estimation and inference for measles outbreaks. Biostatistics. 2002;3:493-510.

30. Fisman DN. Seasonality of infectious diseases. Annu Rev Public Health. 2007;28:127-143.

31. Foege W. House on Fire. The Fight to Eradicate Smallpox. University of California Press; 2011.

32. Gabiane G, Yen PS, Failloux AB. Aedes mosquitoes in the emerging threat of urban yellow fever transmission. Rev Med Virol. 2022;32(4):e2333.

33. Gonzalez-Reiche AS, Hernandez MM, Sullivan MJ, et al. Introductions and early spread of SARS-CoV-2 in the New York City area. Science. 2020;369:297-301.

34. Grassly NC, Fraser C. Seasonal infectious disease epidemiology. Proc Biol Sci. 2006;273:2541-2550.

35. Gray RH, Wawer MJ, Brookmeyer R, et al. Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda. Lancet 2001;357:1149-1153.

36. Greenwood M, Bradford Hill A, Topley WWC. Experimental Epidemiology. His Majesty’s Stationery Office; 1936.

37. Greig JR. Scrapie in sheep. J Comp Pathol. 1950;60:263-266.

38. Grenfell BT, Bjornstad ON, Kappey J. Travelling waves and spatial hierarchies in measles epidemics. Nature. 2001;414:716-723.

39. Grenfell BT, Pybus OG, Gog JR, et al. Unifying the epidemiological and evolutionary dynamics of pathogens. Science. 2004;303:327-332.

40. Griffin DE. Measles virus persistence and its consequences. Curr Opin Virol. 2020;41:46-51.

41. Gussow AB, Auslander N, Faure G, Wolf YI, Zhang F, Koonin EV. Genomic determinants of pathogenicity in SARS-CoV-2 and other human coronaviruses. Proc Natl Acad Sci U S A. 2020;117:15193-15199.

42. Halloran ME, Longini IM Jr, Nizam A, Yang Y. Containing bioterrorist smallpox. Science. 2002;298:1428-1432.

43. Halloran ME, Longini IM Jr, Struchiner CJ. Design and Analysis of Vaccine Studies. Springer; 2010.

44. Halloran ME, Struchiner CJ. Study designs for dependent happenings. Epidemiology. 1991;2:331-338.

45. Heesterbeek JA. A brief history of R0 and a recipe for its calculation. Acta Biotheor. 2002;50:189-204.

46. Hope-Simpson RE. The role of season in the epidemiology of influenza. J Hyg. 1981;86:35-47.

47. Jones BA, Grace D, Kock R, et al. Zoonosis emergence linked to agricultural intensification and environmental change. Proc Natl Acad Sci U S A. 2013;110:8399-8404.

48. Kermack WO, McKendrick AG. Contributions to the mathematical theory of epidemics—I. 1927. Bull Math Biol. 1991;53:33-55.

49. Kindhauser MK, Allen T, Frank V, Santhana RS, Dye C. Zika: the origin and spread of a mosquito-borne virus. Bull World Health Organ. 2016;94:675-686C.

50. Kramarz P, France EK, Destefano F, et al. Population-based study of rotavirus vaccination and intussusception. Pediatr Infect Dis J. 2001;20:410-416.

51. Lessler J, Moss WJ, Lowther SA, Cummings DA. Maintaining high rates of measles immunization in Africa. Epidemiol Infect. 2011;139:1039-1049.

52. Lessler J, Reich NG, Brookmeyer R, Perl TM, Nelson KE, Cummings DA. Incubation periods of acute respiratory viral infections: a systematic review. Lancet Infect Dis. 2009;9:291-300.

53. Liu J, Chen X, Liu Y, et al. Characterization of SARS-CoV-2 worldwide transmission based on evolutionary dynamics and specific viral mutations in the spike protein. Infect Dis Poverty. 2021;10:112.

54. Luke DA, Harris JK. Network analysis in public health: history, methods, and applications. Annu Rev Public Health. 2007;28:69-93.

55. Marks HM. The 1954 Salk poliomyelitis vaccine field trial. Clin Trials. 2011;8:224-234.

56. Martinez ME. The calendar of epidemics: seasonal cycles of infectious diseases. PLoS Pathol. 2018;14:e*1007327*.

57. McLean AR, Anderson RM. Measles in developing countries. Part II. The predicted impact of mass vaccination. Epidemiol Infect. 1988;100:419-442.

58. Metcalf CJE, Wesolowski A, Winter AK, et al. Using serology to anticipate measles post-honeymoon period outbreaks. Trends Microbiol. 2020;28:597-600.

59. Morens DM, Holmes EC, Davis AS, Taubenberger JK. Global rinderpest eradication: lessons learned and why humans should celebrate too. J Infect Dis. 2011;204:502-505.

60. Morris M, Kretzschmar M. Concurrent partnerships and the spread of HIV. AIDS. 1997;11:641-648.

61. Moss WJ, Shendale S, Lindstrand A, et al. Feasibility assessment of measles and rubella eradication. Vaccine. 2021;39:3544-3559.

62. Moss WJ, Strebel P. Biological feasibility of measles eradication. J Infect Dis. 2011;204 suppl 1:S47-S53.

63. Mossong J, Nokes DJ, Edmunds WJ, Cox MJ, Ratnam S, Muller CP. Modeling the impact of subclinical measles transmission in vaccinated populations with waning immunity. Am J Epidemiol. 1999;150:1238-1249.

64. Munoz-Alia MA, Nace RA, Zhang L, Russell SJ. Serotypic evolution of measles virus is constrained by multiple co-dominant B cell epitopes on its surface glycoproteins. Cell Rep Med. 2021;2:100225.

65. Murphy TV, Gargiullo PM, Massoudi MS, et al. Intussusception among infants given an oral rotavirus vaccine. N Engl J Med. 2001;344:564-572.

66. Mutapi F, Roddam A. p values for pathogens: statistical inference from infectious-disease data. Lancet Infect Dis. 2002;2:219-230.

67. Nathanson N. The pathogenesis of poliomyelitis: what we don't know. Adv Virus Res. 2008;71:1-50.

68. Nathanson N, Kew OM. From emergence to eradication: the epidemiology of poliomyelitis deconstructed. Am J Epidemiol. 2010;172:1213-1229.

69. Nathanson N, Martin JR. The epidemiology of poliomyelitis: enigmas surrounding its appearance, epidemicity, and disappearance. Am J Epidemiol. 1979;110:672-692.

70. Neher RA, Bedford T. Real-time analysis and visualization of pathogen sequence data. J Clin Microbiol. 2018;56(11):e00480-18.

71. Nelson MI, Simonsen L, Viboud C, Miller MA, Holmes EC. Phylogenetic analysis reveals the global migration of seasonal influenza A viruses. PLoS Pathol. 2007;3:1220-1228.

72. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team; Dawood FS, Jain S, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med. 2009;360:2605-2615.

73. Panum P. Observations Made During the Epidemic of Measles on the Faroe Islands in the Year 1846. American Public Health Association; 1940.

74. Parvez MK, Parveen S. Evolution and emergence of pathogenic viruses: past, present, and future. Intervirology. 2017;60:1-7.

75. Pitzer VE, Viboud C, Simonsen L, et al. Demographic variability, vaccination, and the spatiotemporal dynamics of rotavirus epidemics. Science. 2009;325:290-294.

76. Pybus OG, Rambaut A. Evolutionary analysis of the dynamics of viral infectious disease. Nat Rev Genet. 2009;10:540-550.

77. Rader B, Gertz A, Iuliano AD, et al. Use of at-home COVID-19 tests—United States, August 23, 2021-March 12, 2022. MMWR Morb Mortal Wkly Rep. 2022;71:489-494.

78. Rambaut A, Pybus OG, Nelson MI, Viboud C, Taubenberger JK, Holmes EC. The genomic and epidemiological dynamics of human influenza A virus. Nature. 2008;453:615-619.

79. Riley S. Large-scale spatial-transmission models of infectious disease. Science. 2007;316:1298-1301.

80. Rogers DJ, Randolph SE. Studying the global distribution of infectious diseases using GIS and RS. Nat Rev Microbiol. 2003;1:231-237.

81. Ross R. An application of the theory of probabilities to the study of a priori pathometry, Part I. Proc R Soc A. 1916;92:204-230.

82. Sartwell PE. The incubation period and the dynamics of infectious disease. Am J Epidemiol. 1966;83:204-206.

83. Shaman J, Galanti M. Will SARS-CoV-2 become endemic? Science. 2020;370:527-529.

84. Shanks GD, Waller M, Briem H, Gottfredsson M. Age-specific measles mortality during the late 19th-early 20th centuries. Epidemiol Infect. 2015;143:3434-3441.

85. Silva-Ramos CR, Faccini-Martinez AA, Calixto OJ, Hidalgo M. Bolivian hemorrhagic fever: a narrative review. Travel Med Infect Dis. 2021;40:102001.

86. Tack DM, Ray L, Griffin PM, et al. Preliminary incidence and trends of infections with pathogens transmitted commonly through food—Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 2016-2019. MMWR Morb Mortal Wkly Rep. 2020;69:509-514.

87. Tamerius J, Nelson MI, Zhou SZ, Viboud C, Miller MA, Alonso WJ. Global influenza seasonality: reconciling patterns across temperate and tropical regions. Environ Health Perspect. 2011;119:439-445.

88. Theze J, Li T, du Plessis L, et al. Genomic epidemiology reconstructs the introduction and spread of Zika virus in Central America and Mexico. Cell Host Microbe. 2018;23:855-864 e7.

89. Truelove SA, Graham M, Moss WJ, Metcalf CJE, Ferrari MJ, Lessler J. Characterizing the impact of spatial clustering of susceptibility for measles elimination. Vaccine. 2019;37:732-741.

90. Weiss RA, McMichael AJ. Social and environmental risk factors in the emergence of infectious diseases. Nat Med. 2004;10(12 suppl):S70-S76.

91. Wesolowski A, Buckee CO, Engo-Monsen K, Metcalf CJE. Connecting mobility to infectious diseases: the promise and limits of mobile phone data. J Infect Dis. 2016;214(suppl 4):S414-S420.

92. Wilkinson AL, Diop OM, Jorba J, Gardner T, Snider CJ, Ahmed J. Surveillance to track progress toward polio eradication—worldwide, 2020-2021. MMWR Morb Mortal Wkly Rep. 2022;71:538-544.

93. Wolfe ND, Dunavan CP, Diamond J. Origins of major human infectious diseases. Nature. 2007;447:279-283.

94. Yang Y, Sugimoto JD, Halloran ME, et al. The transmissibility and control of pandemic influenza A (H1N1) virus. Science. 2009;326:729-733.

95. Yorke JA, Nathanson N, Pianigiani G, Martin J. Seasonality and the requirements for perpetuation and eradication of viruses in populations. Am J Epidemiol. 1979;109:103-123.

96. Zeitz PS, Butler JC, Cheek JE, et al. A case-control study of hantavirus pulmonary syndrome during an outbreak in the southwestern United States. J Infect Dis. 1995;171:864-870.

CHAPTER 14

Antiviral Agents

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Acknowledgments

Overview

Medical Value of Antiviral Drugs and Barriers to Their Development

Viruses are a leading cause of disease and death worldwide. As illustrated most recently by the coronavirus infectious disease of 2019 (COVID-19) pandemic, public health measures and vaccines are the most effective ways to control many viral infections. However, such preventive measures achieve only partial success for numerous viral diseases. For some of these diseases, antiviral drugs have been developed. A number of these drugs have been highly successful, saving lives and relieving suffering. This has been most dramatic with drugs active against human immunodeficiency virus (HIV) and hepatitis C virus (HCV). For HIV, a progressive fatal disease can be transformed into a manageable condition in which virus replication can be suppressed presumably for a lifetime as long as antiretroviral therapy is maintained, and for HCV, a progressive fatal disease can be cured by several weeks of antiviral therapy.

Despite these successes, there are relatively few diseases for which highly effective antiviral drugs have been developed. Several factors contribute to this. Most antivirals are designed to hit a specific viral target, limiting their utility to treat other viral infections. Some viruses (eg, herpesviruses, HIV) establish latent infections, so treating active infections does not cure the patient. Different viruses, especially respiratory viruses, can cause similar symptoms, making diagnosis difficult. Often, treatment must begin early to be beneficial, sometimes because the viral diseases are quickly controlled by immune responses. Finally, many viral illnesses present limited market opportunities that have sometimes discouraged pharmaceutical companies from developing antiviral drugs.

The Process of Antiviral Drug Development

Antiviral drugs can be divided into two classes, one comprising small molecules and, less commonly, peptides and oligonucleotides, and a second comprising macromolecules such as antibodies and interferons. This chapter will cover FDA-approved or -authorized drugs in the former class while the latter agents will be covered in a different chapter in this volume. Older small molecule antiviral drugs such as amantadine and acyclovir (ACV) were discovered by testing chemicals, often synthesized for some other purpose, for their ability to inhibit virus replication in labor- and time-intensive assays of viral replication, such as assays of cytopathic effect or plaque assays.[94,389] This approach has now developed into cell-based high-throughput screens that use automation to allow screening of large compound collections (up to millions of compounds) in a relatively short period of time. One advantage of cellular screening is that it can identify new mechanisms to target for effective antivirals. For example, highly potent compounds that target NS5A, a poorly understood function of HCV, to block HCV replication have been discovered this way.[144]

A more rational approach is to screen large compound collections against a specific viral gene product that would make a good drug target (see below for discussion). Such target-based screens can examine over one million chemical compounds even more rapidly than cell-based screens. The first nonnucleoside reverse transcriptase inhibitors (NNRTIs), which are active against HIV-1, were discovered using this approach (eg, nevirapine[300]).

A structure-based approach allows one to exploit detailed knowledge about a viral protein to design drugs that will inhibit its activity. Knowledge of the 3-dimensional structure of the protein can enable visualization of its active site and understanding of its mechanism of action and can also be used to design or refine small molecules that can block the active site and the protein's action. An example of a drug discovered using this approach is the neuraminidase inhibitor, zanamivir, which is active against influenza A and B virus.[452]

The approaches outlined above result in what is called a "hit"; however, a hit is only an inhibitor of the function of a viral protein or of viral replication in cell culture. The next steps involve using medicinal chemistry to convert the hit into a compound with high potency and desirable pharmacological properties (eg, bioavailability, long elimination half-life, low toxicity), not to mention patentability. If the structure of the hit compound bound to its target is available, this information can be used to help generate additional compounds to be tested. This process will be illustrated below with the example of the anti-HCV NS3/4A inhibitors, the anti-HIV protease inhibitor, ritonavir, and the anti-influenza neuraminidase inhibitors. Simultaneously, studies to demonstrate the mechanism(s) of action of the compounds are undertaken (see section on *General Aspects of Antiviral Drug Mechanisms and Drug Resistance* below). A key but often overlooked aspect of drug development is developing optimal methods for synthesis of pure compound on a large scale and formulation of the compound for administration to humans.

When a candidate compound with desirable properties has been developed, clinical trials begin. There is no rigid formula by which an antiviral candidate is assessed after it enters the clinic. Typically, the process of clinical development starts with the administration of drug in escalating doses to small cohorts of volunteers to assess pharmacokinetics and tolerability (phase I trials). With some virus infections, these studies may be performed in patients in whom drug activity can also be assessed, for example, by quantifying reductions of chronic HCMV shedding in the genitourinary tract or of HCV in the plasma (proof of concept studies). Substantial reductions in viral load encourage progression to more advanced clinical trials.

A major advantage of antiviral drug development, compared to drug development for many other diseases, is that quantitative reductions in virus shedding are a precise measure of activity and in general correlate with clinical endpoints. These quantitative reductions can be measured quickly (days or less) and permit determination of whether drugs work and of appropriate doses and dosing intervals. Phase II clinical trials assess this antiviral activity along with the additional pharmacological and safety information in dozens to several hundred subjects. The data from phase II trials permit the design of larger phase III efficacy studies, which provide statistically robust results that fulfill regulatory requirements for drug approval. Trials for certain antiviral drugs (eg, vs influenza and herpesviruses) require clinical endpoints (ie, amelioration of disease), with virological data supporting those. For HIV, hepatitis B virus (HBV), and HCV, the correlations between levels of viremia and disease and the correlations between drug-induced reductions in viremia and improvement of disease have resulted in the acceptance of virological endpoints (eg, the proportion of patients with viremia below the limit of detection or the proportion with sustained virological response) as criteria for drug approval.

Should the clinical trials yield the desired results, the "hit" will have turned into an approved drug. Even then, its efficacy and safety will be put to the test in much larger populations, and new indications or limitations for its use may emerge.

Importance of Antiviral Drugs for Basic Science

Aside from their importance in treating viral diseases, antiviral drugs make excellent laboratory tools. By blocking functions of specific viral proteins and thus specific stages in viral replication, drugs can be crucial for understanding the timing of particular events in the virus replication cycle and which proteins are needed for each step. Studies using selective antiviral drugs, coupled with viral mutations that confer drug resistance, can help dissect the details of how these proteins function. Drug resistance mutations can provide selectable markers for

engineering interesting mutant viruses and performing viral genetics.

An example of how studies of antiviral drugs and drug resistance can illuminate virus biology and biochemistry is the role of amantadine in the discovery of an influenza A ion channel and its function in virus uncoating.[173,352] Similarly, clinical investigation has been greatly abetted by antiviral drugs. For a recent example, insights into the dynamics of HCV infection have been obtained through detailed analysis of the impact of directly acting antivirals on the kinetics of HCV RNA and liver transaminases in serum.[55]

General Aspects of Antiviral Drug Mechanisms and Drug Resistance

Drug Selectivity, Resistance, and Drug Targets

The value of antiviral drugs for both basic science and therapy depends on how selective they are and on how well their mechanisms are understood. Biologically, selectivity is the difference between the dose of the drug that exerts its antiviral effect and the dose that exerts its toxic effect. These doses are often quantified in cells as the effective concentrations that reduce viral activity and the concentration that causes cytotoxicity, respectively, by 50% (EC_{50} and CC_{50}). However, differences between these values can overestimate therapeutic selectivity, as a 50% reduction in viral replication would have little if any clinical benefit, while a 50% toxic effect could make a drug unsafe for humans. Thus, a more appropriate measure of selectivity is likely to include a dose that reduces viral activity much more drastically; that is, 99% or more. Examples of how selectivity is achieved biochemically can be found among the descriptions of the mechanisms of specific antiviral drugs.

It is generally agreed that the best way to understand antiviral selectivity and mechanism is through the study of drug resistance. The detection of resistance to an antiviral drug implies that the drug is selective. That is, the drug acts at least in part by interfering with a virus-specific process, rather than by incapacitating the host cell.[185] In much the same way, a mutation in a viral gene that confers resistance to an antiviral drug (showing that requires a genetic experiment) implies that the gene or its product is necessary for at least part of the selective action of the drug.

In most cases, drug resistance mutations identify targets of antiviral drugs. However, there are exceptions. For example, anti-influenza virus drugs whose target is neuraminidase (eg, zanamivir) can select for mutations in the gene encoding the hemagglutinin.[164,294,412] These mutations would be expected to result in a hemagglutinin that binds less avidly to cell surface glycoproteins. This in turn could result in a reduced requirement for neuraminidase to release the virion from the cell and thus resistance to the neuraminidase inhibitor. Accordingly, genetics alone is not sufficient to identify a drug target. Ideally, one should also show that the drug interacts with the target, and that the resistance mutation results in a target with an altered interaction with the drug that explains the resistance phenotype. This usually requires biochemical studies, and the analyses are not always straightforward.

Mapping a resistance mutation that results in a single amino acid change in the target protein identifies that residue as being necessary for sensitivity to the drug. Other genetic approaches, such as the generation of chimeric genes from closely related viruses that are susceptible or resistant to a drug (eg, HIV-1 and HIV-2, and NNRTIs[89]), can identify residues that are sufficient to confer susceptibility to the drug to an otherwise resistant protein. However, these results do not necessarily identify residues that interact with the drug. Identification of a drug-binding site requires methods such as photoaffinity cross-linking and X-ray crystallography. These two methods identified the binding site for the NNRTI, nevirapine, on HIV-1 RT.[86,220,478] Mapping a drug-binding site is valuable but does not tell us how drug binding exerts its physiological action. In the case of NNRTIs, presteady state kinetic analyses of HIV-1 RT were used to show that the drug slows the enzyme's incorporation of dNTPs into DNA,[373,408] presumably by altering the orientation of catalytic residues, as suggested by the location of the drug-binding site.[220] Thus, solving the mechanism of action of an antiviral drug requires multidisciplinary approaches, and in some cases, we still lack a complete understanding.

General Aspects of Antiviral Drug Targets

There are two major kinds of virus proteins that serve as targets for small molecule antiviral drugs. As, in general, it is easier to inhibit a protein's function (with an antagonist) than to promote its function (with an agonist), the most common antiviral drug target is a virus-encoded protein that can be inhibited by a drug. Ideally, the target is essential for viral replication. Less ideally, it should at least be very important for replication and pathogenesis in the human host. The target should also be sufficiently different from any important host proteins to permit selectivity. Ideally, it should be *druggable*; that is, a small molecule should be able to inhibit it. Enzymes make especially good targets for inhibition, because they are usually at low concentrations inside cells, they are relatively well understood mechanistically, they often interact with small-molecule substrates, and, as catalysts, their inhibition interrupts multiple reactions. A second kind of target, which does not need to be essential, is a virus-encoded protein that can activate the drug to make it inhibitory to the virus (*lethal synthesis*), and that is sufficiently different from host counterparts to permit selectivity. An example is HSV thymidine kinase (TK), which activates ACV and its congeners.[142]

Host proteins may also serve as antiviral drug targets, if they have an activity that is more important for viral replication than for host health. However, targeting host functions is inherently more likely to result in toxicity than targeting viral functions. Additionally, as noted above, given that antiviral efficacy requires orders of magnitude effects on viral replication while meaningful toxicity often only requires twofold effects, there is a high barrier to developing safe and effective antiviral drugs against host targets. Despite that, there are successful examples of this approach in targeting cell surface molecules. The prime example is the host target, CCR5, which led to the development of the anti-HIV drug maraviroc.[248,357] This example is a testament to the value of human genetic studies of subjects carrying a null mutation in the gene encoding CCR5, who remained HIV negative and in good health despite frequent exposure to the virus.[268] However, most host functions do not show such a dichotomy.

It has been argued that viruses should be less likely to develop resistance against drugs that target host functions. Regardless, such mutants certainly can develop (eg, see Refs.[390,442]).

Factors Affecting the Development of Drug Resistance

If a drug acts selectively against a virus, the virus will generally be able to develop resistance to it. However, the frequency of drug resistant mutants in a population and how rapidly they arise depend on several factors: the first is the mutation rate of the virus. The higher the mutation

rate, the more rapidly resistance can develop. Viral mutation rates are largely controlled by the fidelity of the polymerases that replicate the viral genomes. RNA viruses, which replicate using low fidelity RNA polymerases, usually have the highest mutation rates, averaging one mutation per genome per replication cycle,[106,281] while DNA viruses, whose DNA polymerases include proofreading 3′-5′ exonucleases that contribute to fidelity,[199] have lower mutation rates. However, viral and host DNA repair proteins and other factors, including nucleotide pools, which can be modulated by both viral and cellular factors, also affect mutation rate.

The second factor is the target size for mutation. The more sites where mutations can confer drug resistance, the more rapidly resistance can arise. For some drugs, such as ACV, many mutations can confer resistance, as any mutation that substantially reduces viral TK activity results in resistance. Also, some sites within a gene are more or less likely to mutate than others. Homopolymeric runs in the HSV *tk* gene (eg, a run of 7 G's) are hot spots for frameshift mutations that confer ACV resistance.[197]

The third factor is magnitude of replication of the virus. The more copies of viral genomes produced, the more opportunities for resistance to arise. The fourth factor is the preexisting size of the virus population. The more virus present, even in the absence of drug selection, the more likely that drug-resistant mutants will be present among the array of genetic variants. Examples of these two factors that have an impact on HIV drug resistance are provided below in the section on *Drug Resistance in Antiviral Therapy*.

The fifth factor involves fitness (ie, how well a genetic variant reproduces relative to other genetic variants, which can include "wild type"). The fitter the drug resistant mutant, the likelier it is to emerge during treatment. For a virus to cause disease that is resistant to an antiviral drug, it must mutate not only to evade drug action but also to retain pathogenicity. Some drug resistant mutants, such as influenza viruses that are resistant to adamantanes, can be highly fit in cell culture, animal models,[29,427] and in humans (reviewed in Ref.[174]). On the other hand, HCV mutants resistant to sofosbuvir replicate very poorly in cell culture, and this has translated to a major advantage for the clinical use of this drug.[234,244,426] (This illustrates the advantage of designing antivirals to interact with functional sites on essential targets.) Many drug-resistant herpesvirus mutants are highly fit in cell culture but are much less so in animal models, which correlates with the low frequency that these mutants are recovered in immunocompetent humans, while others are less fit in cell culture, yet not so impaired that they do not cause disease in patients[25] (reviewed in Refs.[82,239]). Relative fitness is important both in the absence and the presence of drug. When treatment is stopped, if drug-resistant mutants are less fit in that setting, wild-type virus can return to predominate. These various factors—mutation rate, target size, replication rate, preexisting population, and fitness—can be taken into account in mathematical formulae and used to model the emergence of drug resistance (eg, see Ref.[85]).

These factors also influence how different viral infections are treated. For example, HIV infections are now treated with combinations of antiviral drugs, while HSV infections are treated with single agents. This will be discussed below in the sections on antiviral therapy.

Mechanisms of Specific Antiviral Drugs

This section, with a few exceptions, will consider agents currently authorized or approved by the FDA and marketed for clinical use. No endorsement of the therapeutic value of these drugs is intended. For certain viruses that can be studied usefully in animal models, we will discuss the effects of drug resistance mutations on replication and pathogenesis *in vivo*.

Targeting Drugs to Specific Stages of Virus Infection

As detailed throughout this book, the infection of cells by viruses can be broken down into a common set of steps or stages. Most of these stages have been targeted by approved antiviral drugs; for this chapter, we have divided the stages into entry, uncoating, gene expression, genome replication, and assembly/egress (Fig. 14.1). The order of stages can vary for different viruses, and some viruses have steps within stages that are not shared with other viruses. For example, the replication of HIV and the steps that have been targeted by approved antiviral drugs are illustrated in Figure 14.2. In principle, any stage can be targeted for inhibition. A potential advantage to targeting very early steps such as attachment during the entry stage, or very late steps, such as release during the assembly/egress stage, is that drugs, such as certain peptides, that act at these steps do not have to enter cells to exert activity (most orally available drugs enter cells readily). Stages such as gene expression, genome replication, and assembly/egress often require specific viral enzymes, which, as described above, are attractive drug targets. Indeed, small molecules that inhibit genome replication comprise the largest class of antiviral drugs.

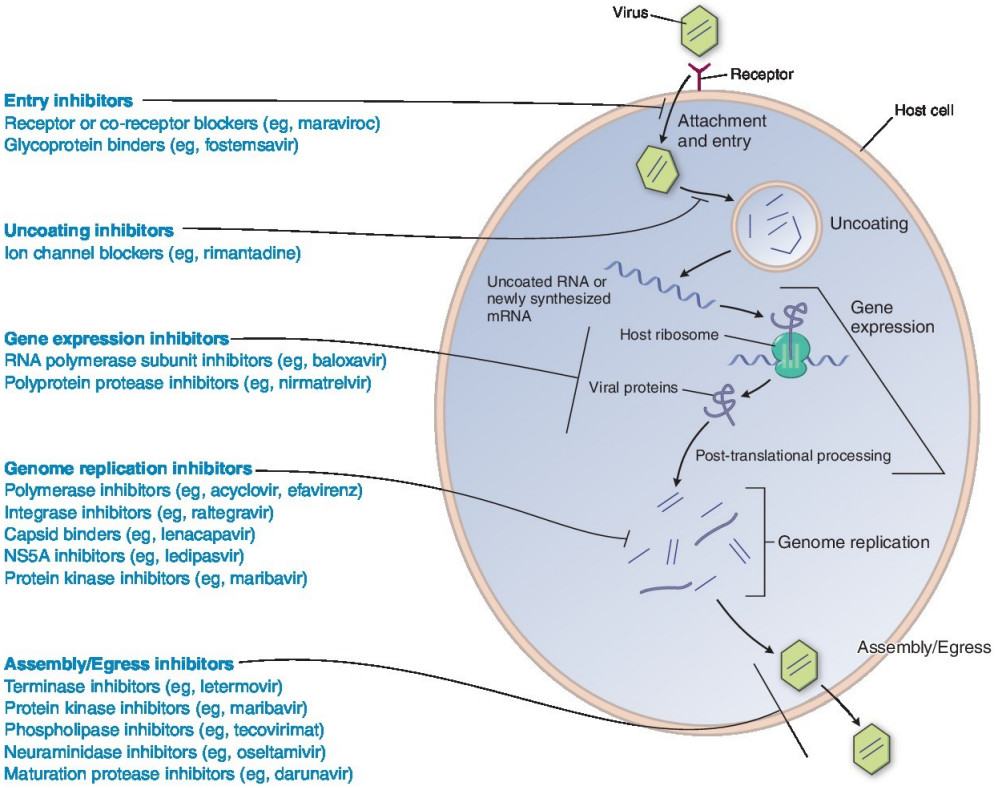

FIGURE 14.1 **Antiviral drugs block various stages of viral replication**. A generic replication cycle of viruses in cells is cartooned, showing the stages of infection, which different drug classes block. These include entry, uncoating, gene expression, genome replication, and assembly/egress. Classes of drugs with examples are provided. For some viruses (eg, HIV), the order of the stages differs from that in this cartoon (see Fig. 14.2, which also highlights different steps within stages). (Modified with permission from Li JZ, Coen DM. Pharmacology of viral infections. In: Golan DE, Armstrong EJ, Armstrong AW, eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. 4th ed. Wolters Kluwer; 2017:694-722, Ref.[255])

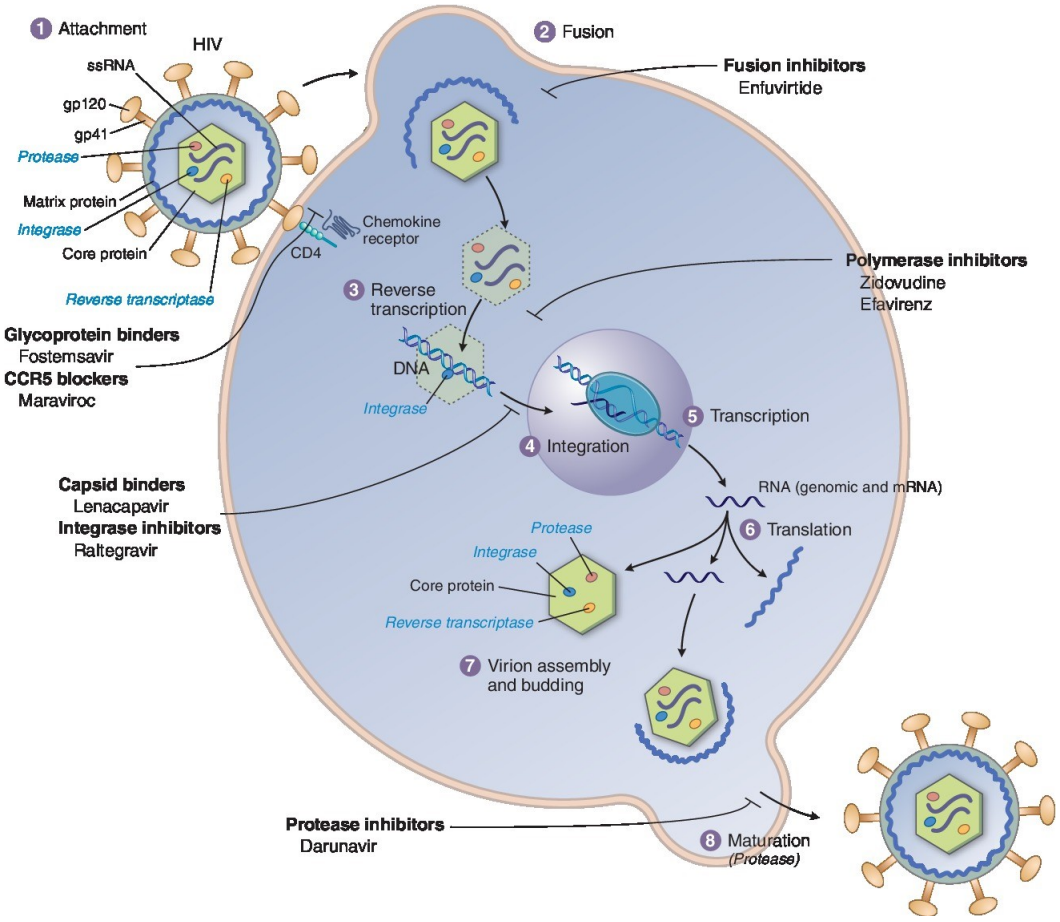

FIGURE 14.2 **Steps of HIV infection blocked by different classes of antiviral drugs**. The life cycle of HIV is cartooned, showing: **1**. Virus attachment is dependent on binding interactions between viral gp160 (composed of gp41 and gp120 proteins) and host cell CD4 and certain chemokine receptors. **2**. Fusion of the viral membrane (envelope) with the host cell plasma membrane allows the HIV genome complexed with certain virion proteins to enter the host cell. **3**. Uncoating permits the single-stranded RNA (ssRNA) HIV genome to be copied by reverse transcriptase into double-stranded DNA. **4**. The HIV DNA is integrated into the host cell genome, in a reaction that depends on HIV-encoded integrase. **5**. Gene transcription and posttranscriptional processing by host cell enzymes abetted by viral gene products produce genomic HIV RNA and viral mRNA. **6**. The viral mRNA is translated into proteins on host cell ribosomes. **7**. The proteins assemble into immature virions that bud from the host cell membrane. **8**. The virions undergo proteolytic cleavage, maturing into fully infective virions. The steps at which classes of anti-HIV drugs act (attachment, fusion, reverse transcription, integration, and maturation) are indicated, with examples from specific drug classes provided. (Adapted with permission from Li JZ, Coen DM. Pharmacology of viral infections. In: Golan DE, Armstrong EJ, Armstrong AW, eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. 4th ed. Wolters Kluwer; 2017:694-722.)

Inhibition of Viral Entry

Inhibition of entry prevents all subsequent steps in virus infection and permits the virion to be cleared by immune and other mechanisms. Mechanisms by which viruses enter host cells are reviewed elsewhere in this edition. Briefly, the first step is attachment wherein one or more specific proteins on the virion surface (eg, HIV glycoprotein [gp] 120) bind to one or more specific receptor molecules (eg, for HIV, CD4 and coreceptors CCR5 or CXCR4) on the cell surface. These receptors are usually proteins but are sometimes simpler entities such as modifying groups (eg, sialic acid for influenza virus) on cell surface molecules. The viral protein-host receptor interactions can result in changes in one or both entities. Entry then requires that nucleocapsids cross host membranes to enter the cytoplasm, sometimes directly across the plasma membrane and sometimes following internalization of the virion bound to the receptor. In the case of enveloped viruses such as HIV, entry involves fusion of the viral envelope (membrane) with a host membrane, which often entails dramatic conformational changes (this process for HIV is cartooned in Fig. 14.3).

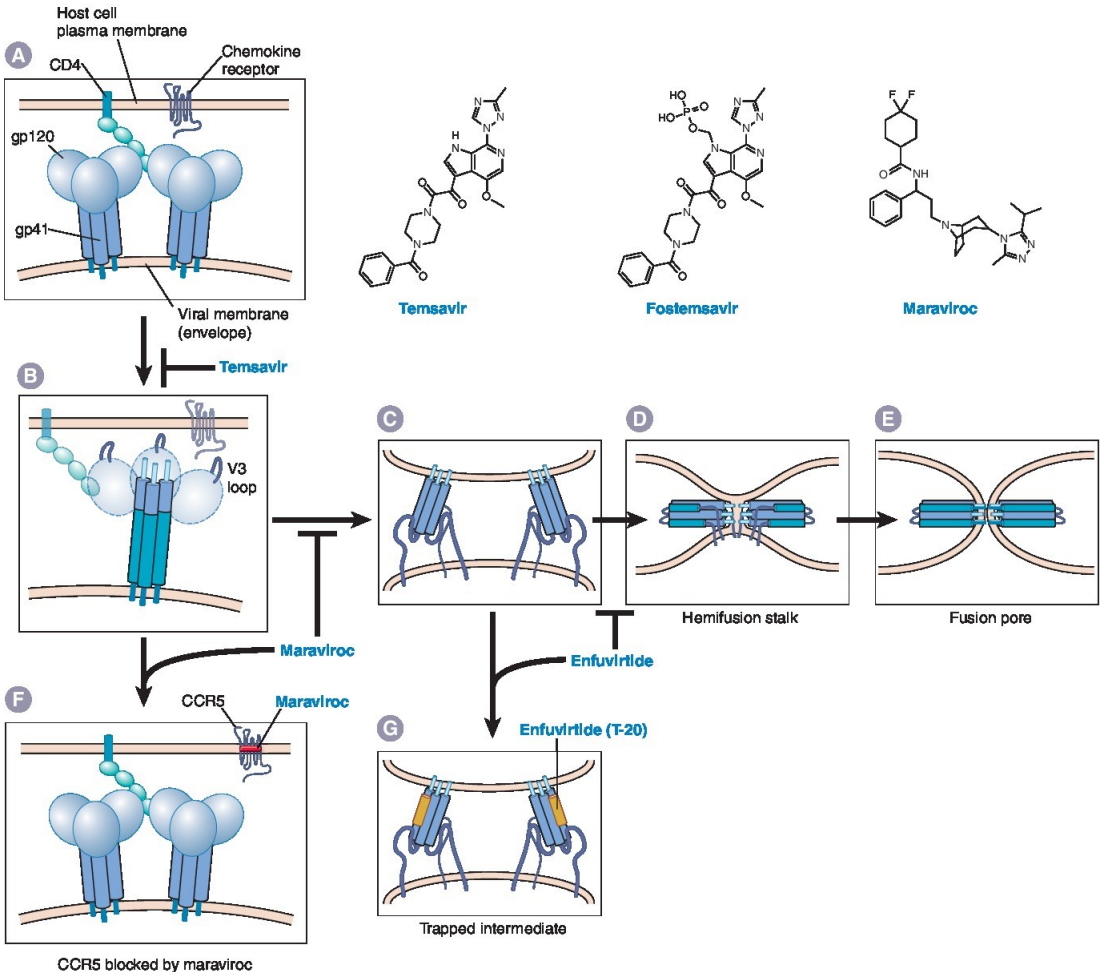

FIGURE 14.3 **Model for HIV gp41–mediated fusion and drug action**. **A**. HIV glycoproteins exist in trimeric form in the viral envelope. Each gp120 molecule is depicted as a ball attached noncovalently to gp41. The CD4 receptor and a chemokine receptor that serves as a coreceptor in the host cell plasma membrane are shown. The structures of temsavir, its orally available form, fostemsavir, and maraviroc are depicted. **B**. The binding of gp120 to CD4 causes a conformational change that results in the V3 loop of gp120 contacting the coreceptor. Temsavir binds to gp120 and blocks this conformational change. Binding of gp120 to the coreceptor causes a conformational change in gp41 that exposes the fusion peptide, heptad-repeat region 1 (HR1) and heptad-repeat region 2 (HR2). The fusion peptide inserts into the host cell plasma membrane. Maraviroc binds to one of the chemokine receptors that can serve as a coreceptor, CCR5, and can block the gp120-CCR5 interaction and thus the gp41 conformational change of HIV strains that use CCR5. **C**. gp41 undergoes further conformational changes, characterized by unfolding and refolding of the HR2 segments. Enfuvirtide is a synthetic peptide that mimics HR2 and binds to HR1. It prevents the HR1-HR2 interaction and blocks further refolding. **D**. Completed refolding of

the HR regions creates a hemifusion stalk in which the outer leaflets of the viral and host cell membranes are fused. **E**. Formation of a complete fusion pore allows viral entry into the host cell. **F**. Maraviroc traps the virus-host cell interaction at the attachment stage, preventing subsequent conformational changes, membrane fusion, and viral entry. **G**. Enfuvirtide traps gp41 so that HR2 cannot fold onto HR1, thereby blocking hemipore formation and fusion. (Adapted with permission from Li JZ, Coen DM. Pharmacology of viral infections. In: Golan DE, Armstrong EJ, Armstrong AW, eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. 4th ed. Wolters Kluwer; 2017:694-722.)

Drugs that inhibit entry can target either viral proteins or host proteins. Thus far, efforts to find such drugs have resulted in three approved anti-HIV drugs, fostemsavir, maraviroc, and enfuvirtide (plus a monoclonal antibody drug, ibalizumab), and a drug active against hepatitis B and D viruses (HBV and HDV), bulevirtide, that at this writing is poised for approval. Three of these drugs have unusual properties: maraviroc and bulevirtide target a host protein, and bulevirtide and enfuvirtide are peptides.

Blocking Entry With a Viral Glycoprotein-Derived Peptide: Bulevirtide

HBV and HDV are enveloped viruses displaying three HBV-encoded glycoproteins, S, M, and L, on their surfaces. HDV requires HBV for its propagation; infection with both HBV and HDV causes more serious disease than with HBV alone. A specific segment of HBV glycoprotein L called the pre-S1 domain, which is myrisotylated at its N-terminus, is necessary for infectivity of HBV and HDV, and peptides derived from it are sufficient to bind to liver cells.[254,295,317,422] Pretreatment of cells with myristoylated peptides corresponding to roughly the first half of this segment can specifically inhibit HBV and HDV infectivity with low nM potency, and replacement of the myristate with longer fatty acids resulted in even greater potency.[27,116,161] Starting with a myristoylated pre–S1-derived peptide modified to permit photocrosslinking, Yan et al. identified a host cell protein, sodium taurocholate cotransporting polypeptide (NTCP), as the virus receptor.[482] A myristoylated pre-S peptide can block binding of HDV to cells expressing NTCP[482] suggesting a block at the step of attachment, although effects on subsequent steps leading to virus entry cannot be ruled out. NTCP is crucial for the transport of bile acids, and human genetic studies have indicated that mutations disrupting NTCP function result in substantially elevated serum levels of bile; however, there appears to be much less risk to health than HBV and HDV disease.[449] These findings led to the synthesis and development of an injectable 47 residue peptide (residues 2-48 of glycoprotein L) with an N-tetradecanoylglycyl–modified N-terminus and an amidated C-terminus (bulevirtide), which, at this writing, has been approved in Europe and is under review by the FDA for treatment of chronic HDV infection with compensated liver disease. Given that bulevirtide engages a host protein, one might expect resistance to be unlikely, but not impossible. As of 2020, no resistance to bulevirtide had been detected in treated patients.[123] Continued *in vitro* and clinical studies will be needed to address this possibility.

Targeting a Viral Glycoprotein With a Small Molecule: Fostemsavir

Fostemsavir (Fig. 14.3) was developed starting from a hit that came out of a cell-based screen for compounds that would block an HIV-derived pseudovirus from infecting cells and expressing a reporter gene.[260] Follow-up studies found that the hit compound acted very early in infection and could inhibit the ability of cells expressing the HIV glycoprotein gp120 to fuse with cells expressing CD4 and CCR5.[260] After lengthy medicinal chemistry, preclinical and clinical efforts, as well as studies[166,260] showing that active compounds bind to gp120, and that specific mutations that alter gp120 both reduce binding and confer resistance, the hit was developed into fostemsavir, which is phosphonooxymethyl prodrug of the active 6-azaindole piperazine, temsavir (Fig. 14.3).

Although temsavir and its analogs are frequently described as attachment inhibitors, their mechanism is controversial. Early studies showed that one of these compounds could inhibit binding of gp120 to soluble CD4, supporting a block to attachment.[260] However, the 50% inhibitory dose (IC_{50}) in this assay was ~10-fold higher than in an assay measuring effects on viral replication,[260] and another study found little or no effect on gp120-CD4 binding.[400] Moreover, it was reported that the compounds remain active against HIV strains that can enter cells that don't express CD4.[258] Detailed crystallographic, biochemical, and mutational studies then determined that when members of this class of compounds bind gp120, they interact with a crucial beta-hairpin to stabilize a conformation of gp120 that is still able to bind CD4, but unable to undergo conformational changes that permit engagement of coreceptors that eventually lead to the fusion step of entry (eg, see Refs.[186,342,400]) (Fig. 14.3). It appears then that temsavir has activity at the attachment step, but that its major mode of action is at the subsequent step involving conformational changes and coreceptor interactions.

As noted above, mutations altering gp120 can cause resistance to temsavir in infected cells. As certain of these mutations have been found in patients treated with fostemsavir, they clearly retain some fitness.[226]

Targeting a Coreceptor: Maraviroc

Maraviroc (Fig. 14.3) inhibits binding of HIV to the CCR5 coreceptor. CCR5 is the coreceptor used by the most commonly transmitted HIV-1 strains (R5 tropic), which predominate during the early stages of HIV-1 infection.[47] There are several attractive features to CCR5 as a target for anti-HIV drugs: knockout mice lacking CCR5 and humans with homozygous deletions in the gene encoding CCR5 (CCR5-Δ32) remain healthy albeit with some immune deficits in countering infections other than HIV, suggesting that blocking this host function would not have consequences anywhere near as adverse as HIV disease.[56] Certain of these CCR5Δ32 homozygotes who have been highly exposed to HIV have remained uninfected, and their cells are resistant to R5-tropic strains of HIV *in vitro*.[268] Blockade of CCR5 with a natural ligand (eg, RANTES) or antibody inhibits R5-tropic HIV replication (eg,[81]). These observations suggested that blocking CCR5 could be efficacious against R5-tropic HIV without causing serious toxicity.

Maraviroc was discovered using high throughput screening for small molecules that block binding of CCR5 to a natural ligand, followed by medicinal chemistry efforts to improve upon a hit from the screen.[102] Modeling and mutational studies suggest that maraviroc and several other CCR5 blockers bind to a pocket on CCR5 formed by transmembrane domains,[224] and allosterically inhibit binding to HIV gp120, thereby blocking HIV entry (Fig. 14.3).[17,102,146,460] R5-tropic HIV mutants resistant to CCR5 blockers including maraviroc contain altered gp120 molecules that are able to bind to the drug-bound form of CCR5[442,460] and are often highly fit.[8] Maraviroc was approved in 2007 for use in combination with other anti-HIV drugs in patients with R5-tropic strains. Thus, patients must be tested to determine if they harbor virus that utilizes a different coreceptor (ie, CXCR4). Maraviroc treatment failures are more frequently attributable to preexisting minority populations of CXCR4-tropic virus than to selection of R5-tropic virus that is resistant to maraviroc.[308] Resistance to CCR5 blockers has not resulted thus far

from R5-tropic virus evolving to use a different coreceptor. Nevertheless, this possibility and the effects of antagonizing a host function remain potential issues for the long-term clinical use of maraviroc.

A Fusion Inhibitor: Enfuvirtide

Enfuvirtide was discovered by a rational, directed approach that examined the ability of peptides to inhibit HIV infection in cell culture.[467,468] The peptide that was most potent, enfuvirtide, is similar to a segment of gp41, the HIV protein that mediates membrane fusion. The proposed mechanism for gp41-mediated membrane fusion and enfuvirtide action is illustrated in Figure **14.3**: The gp41 protein in the virion is ordinarily trapped in a metastable conformation so that it cannot fuse membranes or bind enfuvirtide. Binding of HIV to CD4 and then to a coreceptor triggers conformational changes that expose the segment of gp41 thought to insert into host cell membranes (fusion peptide), a helically coiled heptad repeat region (HR-1), and the segment that enfuvirtide resembles, which is a second helical heptad repeat (HR-2). The trimeric gp41 then rearranges, so that the HR-1 and HR-2 helices bind to each other, forming a six-helix bundle. If the fusion peptide has inserted into the host cell membrane, this refolding brings the virion envelope and the cell membrane in proximity, allowing membrane fusion to occur (by mechanisms that remain incompletely understood). When enfuvirtide is present, however, it binds to HR-1 and prevents the refolding process, thereby preventing fusion of the HIV envelope with the host cell membrane.

Although this model requires an exogenous peptide to compete with an intramolecular interaction to bind to a target that is available only transiently, it is supported by a number of observations. These include the crystal structure of the rearranged form of gp41, which shows the HR-1-HR-2 interaction[59,432,458] and evidence that enfuvirtide interacts with HR-1 containing peptides.[60,247,466] Genetic studies show that HR-1 and HR-2 are crucial for fusion.[107,465] Perhaps the strongest evidence that enfuvirtide inhibits HIV replication by binding to HR-1 is that HIV mutations that confer resistance to enfuvirtide alter HR-1 residues, and that these alterations weaken binding of enfuvirtide to HR-1.[372] A number of such mutants exhibit reduced fitness in cell culture, although they have been recovered from patients.[271]

Inhibition of Viral Uncoating: Adamantane Derivatives

The adamantanes, amantadine and rimantadine (Fig. 14.4), were the first successful anti-influenza drugs, although they are no longer recommended for use due to widespread resistance of circulating strains and the availability of more effective alternatives. The adamantanes are active exclusively against influenza A virus (and not influenza B, C, or D viruses) and inhibit virus uncoating. The key to understanding their mechanism was discovered by Hay and colleagues[173] who showed that mutants that could replicate at therapeutic concentrations of amantadine were resistant due to point mutations within the M segment of the influenza RNA genome, in an open reading frame that had only recently been recognized. This open reading frame encodes a small membrane protein known as M_2 (the M segment also encodes the matrix [M_1] protein.) Subsequent biochemical and electrophysiological experiments showed that the M_2 protein forms a tetrameric, pH-gated channel for H^+ ions and that amantadine and rimantadine block this channel.[32,108,189,352,415] Viral mutations conferring resistance to these drugs alter any of five residues in the transmembrane segment of the protein that are predicted to be within the pore of the channel, suggesting that the drugs bind to these residues and "plug" the channel.[172] Structural studies of drug bound versions of M_2, electrophysiological assays using Xenopus oocytes, and assays of engineered viral mutants support this suggestion.[53,206,327,414]

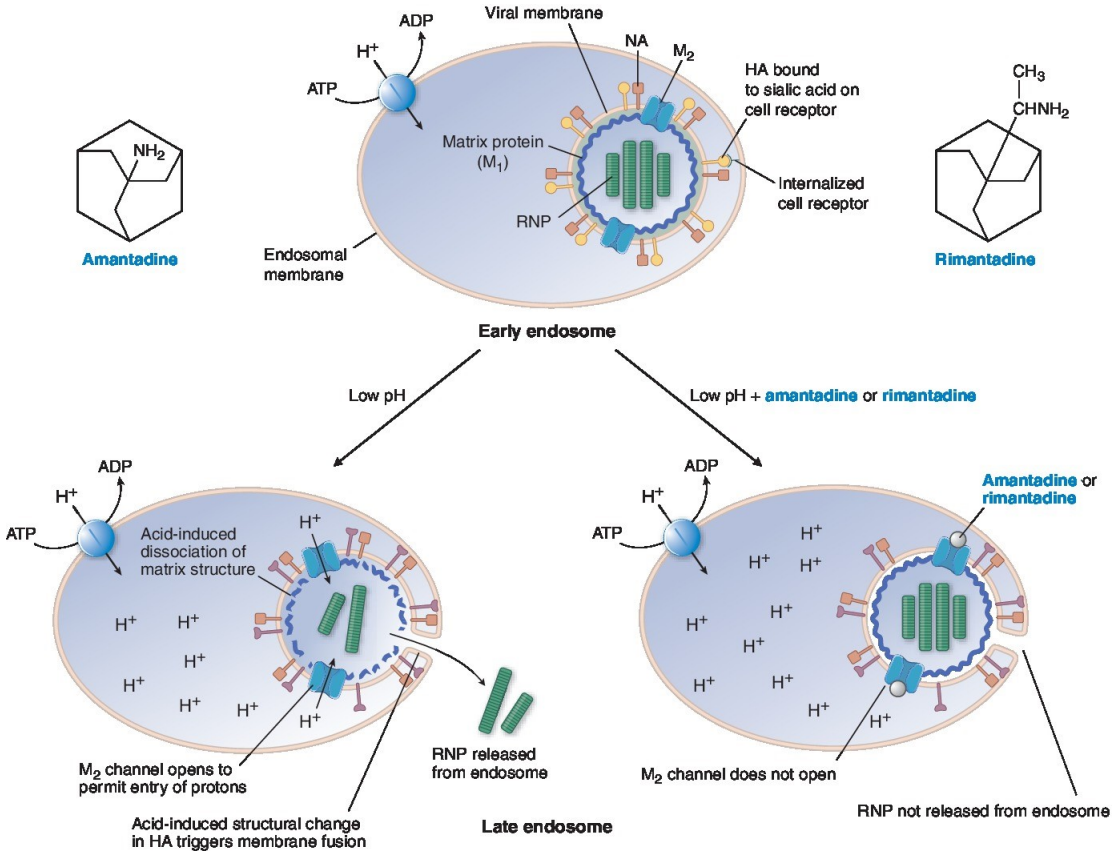

FIGURE 14.4 **Model for uncoating of influenza A virus and effect of amantadine or rimantadine**. The structures of

amantadine and rimantadine are shown. The process of influenza A virus uncoating and drug action is depicted. The virus enters host cells by receptor-mediated endocytosis (not shown) and is contained within an early endosome. The early endosome contains a H^+-ATPase that acidifies the endosome by pumping protons from the cytosol into the endosome. A low pH–dependent conformational change in the envelope protein, hemagglutinin (HA), triggers fusion of the viral membrane with the endosomal membrane. This fusion event, however, is not sufficient to cause viral uncoating. In addition, protons from the late endosome must enter the virion through the M_2 proton channel, which is a virion envelope protein that opens in response to acidification (pH-gated ion channel). The entry of protons into the virion causes dissociation of the M_1 matrix protein from the influenza A ribonucleoprotein (RNP), releasing it into the cytoplasm. The dissociation of matrix is required for RNP to be transported to the nucleus, where it is transcribed and replicated. Amantadine and rimantadine block the M_2 ion channel and thereby inhibit acidification of the interior of the virion, matrix dissociation, and uncoating. NA, neuraminidase. (Adapted with permission from Li JZ, Coen DM. Pharmacology of viral infections. In: Golan DE, Armstrong EJ, Armstrong AW, eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. 4th ed. Wolters Kluwer; 2017:694-722.)

How would blocking an ion channel lead to inhibition of uncoating? A reasonable but not completely verified model is diagrammed in Figure 14.4. Influenza A virus attaches to sialic moieties on cell surface glycoproteins, and then, bound to its receptor, is internalized, surrounded by a cell membrane-bounded compartment, the endosome, which normally participates in membrane protein recycling. As part of its normal function, the endosome becomes acidified. During influenza virus entry, this reduction in pH causes a conformational change in the virion hemagglutinin protein and fusion of the virion envelope and the endosomal membrane via a mechanism similar to that of HIV gp41 (Fig. 14.3). By itself, this action would release viral ribonucleoprotein into the cytoplasm. However, in the presence of amantadine or rimantadine, the matrix protein, M_1, does not dissociate from the ribonucleoprotein, which remains in the cytoplasm instead of entering the nucleus.[51,286] Low pH can promote the dissociation of M_1 and allow nuclear entry of the ribonucleoprotein.[50] Thus, M_2 in the virion envelope functions to let H^+ ions from the acidified endosome enter the virion and dissociate M_1 from the ribonucleoprotein. Amantadine and rimantadine would block the entry of H^+ ions, thereby preventing this uncoating event.

With certain influenza A virus strains, amantadine and rimantadine also block late events in virus replication, in particular, maintenance of hemagglutinin in a proper conformation for infectivity.[172]

Influenza virus mutants that are resistant to amantadine and rimantadine due to altered M_2 have been assayed in animal models including mice and ferrets and show little or no diminution in their replication or pathogenesis.[29,336,427] This correlates well with their behavior in human infections.[174]

Inhibition of Viral Gene Expression

Viral gene expression entails not only the transcription of the viral genome into mRNA, the translation of mRNA into protein, and the stabilities of RNA and protein gene products but can also include a variety of processing events including capping, splicing, and polyadenylation of viral mRNAs, and proteolytic cleavage of viral polyproteins into their individual protein units. Many viruses encode functions that execute or abet one or more of these steps in gene expression, and much effort has gone into targeting these functions for development of antiviral drugs. One such function is performed by the cap-snatching endonuclease subunit of influenza virus RNA polymerase, which is crucial for synthesis of viral mRNAs and is targeted by baloxavir. A second function is performed by viral proteases that generate individual viral proteins. This function has been targeted by antiviral drugs against HIV, HCV, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the cause of COVID-19. The activity of one of these, the HIV protease, is not required until the viral particle is fully assembled, and thus will be discussed under *Inhibition of Viral Assembly/Egress* (see below). The activities of the HCV NS3/4A protease and the SARS-CoV-2 main (M) protease, however, are required for proper expression of viral gene products for essentially all subsequent steps in viral infection, and successful antiviral drugs have been developed against these enzymes.

Inhibition of Influenza mRNA Transcription: Baloxavir

Following uncoating, each segment of the influenza virus negative strand genome (vRNA) enters the nucleus associated with a copy of the virally encoded, heterotrimeric (PB1, PB2, and PA subunits) RNA-dependent RNA polymerase (RdRp).[103,436] The RdRp is responsible both for replication of the viral genome and for transcription to generate mRNAs required for viral protein translation. Influenza mRNA production employs a “cap-snatching” mechanism whereby 5′ 7-methyl guanosine–capped host transcripts are captured and cleaved to generate short-capped RNAs that act as primers for transcription (Fig. 14.5): A capped host transcript is first recognized and bound by the PB2 subunit of the influenza polymerase (Fig. 14.5A). The transcript is then cleaved 10-13 residues from the 5′ cap by a cap-dependent endonuclease (CEN) encoded within the PA subunit[103,227,353] (Fig. 14.5A). This capped fragment then acts as a primer, which is elongated by the PB1 subunit in a template-dependent fashion to generate viral mRNAs complementary to the genomic RNA segment to which the RdRp is bound (Fig. 14.5B-E). This mechanism also ensures that influenza mRNAs are capped, so that they are stable and can be translated efficiently by the host translational machinery.

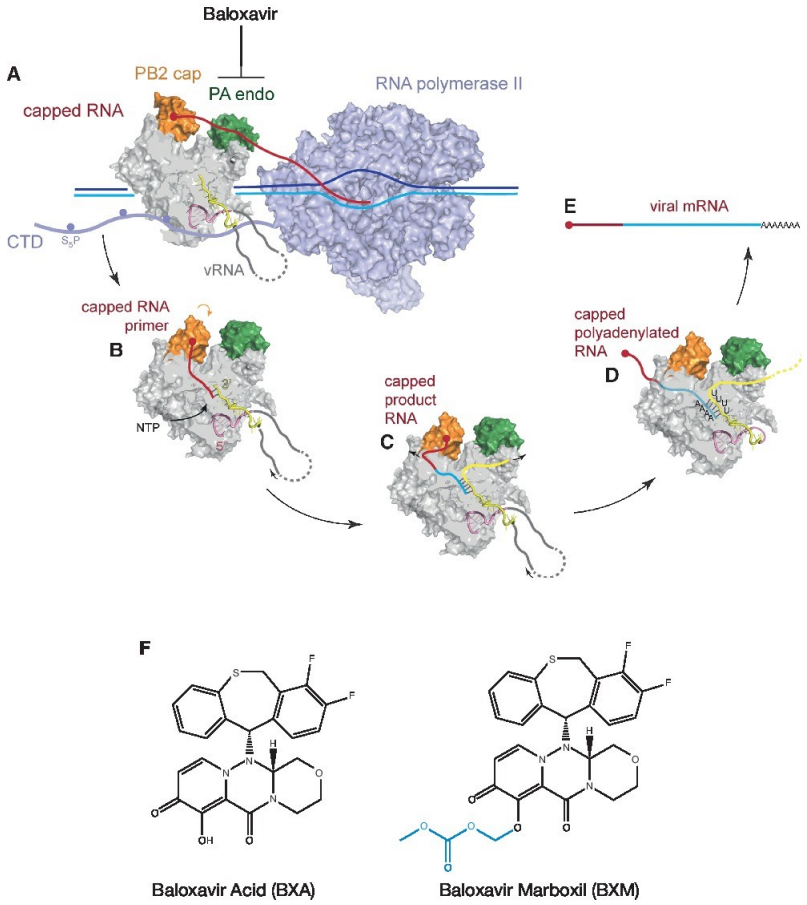

FIGURE 14.5 **Baloxavir, an inhibitor of the cap-snatching step of influenza transcription**. **A**. The three-subunit influenza polymerase (PA, PB1, PB2) associates with a nascent host mRNA and host RNA polymerase II, which permits binding of the host mRNA cap to the PB2 cap-binding domain and cleavage of the nascent transcript by the PA endonuclease (endo). Baloxavir inhibits the endonuclease. **B-E**. In the absence of baloxavir, the cleaved 5′ capped end of the nascent host transcript serves as a primer for the PB1 RNA polymerase, which is bound to viral genomic RNA (vRNA) and elongates the primer in a template-dependent manner, and then polyadenylates the transcript by stuttering on an oligouridine stretch in the vRNA to yield the completed influenza mRNA. **F**. The structures of baloxavir acid, the active drug, and baloxavir marboxil, the prodrug. (Adapted from Krammer F, Palese P. Orthomyxoviridae: The viruses and their replication. In: Howley PM, Knipe DM, eds. *Fields Virology: Emerging Viruses*. Vol 1. 7th ed. Wolters Kluwer; 2021:596-648.)

Baloxavir acid (BXA; Fig. 14.5F) is an inhibitor of the essential CEN function of influenza A and B PA, hence preventing mRNA transcription.[323,440] It was designed to mimic the mechanism of inhibitors of HIV integrase strand transfer (see under *Inhibition of Viral Genome Replication* below), as both enzymes require two divalent cations for their endonuclease function.[323] Critical to the compound design was retention of a di-ketophenol of the oxazino-pyridotriazin-dione (Fig. 14.5F), which acts as a metal chelation site, binding the two required divalent cations within the endonuclease active site.[330] The second “wing” of the molecule (difluoro-dihydro-dibenzothiepine; Fig. 14.5F) makes extensive van der Waals contacts with hydrophobic residues in the active site (eg, Ala20 and Tyr24, Lys34, Ala37, and Ile38 for influenza A).[330,485] BXA demonstrates a low nM IC_{50} for inhibition of endonuclease activity, with no measurable activity on CEN-independent RdRp activity, or human pol II.[323] BXA shows similar potency in inhibiting influenza A and B replication and in decreasing mRNA, complementary RNA for genome replication, and vRNA synthesis in infected cells,[323,330] consistent with a requirement for cap snatching to prime mRNA synthesis that is required for subsequent genome replication.[350]

Studies of mutants derived by *in vitro* selection or emergent resistance in the clinic demonstrate substitutions at isoleucine 38 as conferring resistance to BXA. X-ray crystallographic studies demonstrate that I38 is in the hydrophobic pocket bound by the difluoro-dihydro-dibenzothiepine wing on the molecule.[330] The I38T substitution increased BXA EC_{50} for *in vitro* influenza replication 30-50 fold, with smaller diminutions in activity noted for other position 38 substitutions.[179,323] Acquisition of resistance clinically, correlated with an increase in the median time to symptom resolution.[179] Further characterization of the I38T substitution showed a decreased endonuclease activity as well as *in vitro* replicative viral fitness, potentially through decreased RNA binding.[323,330,485]

To improve oral bioavailability, baloxavir acid is administered as a prodrug, baloxavir marboxil, (BXM) (Fig. 14.5F).

Inhibition of Polyprotein Cleavage: HCV NS3/4A Protease Inhibitors

As noted above, proteolytic cleavage of viral polyproteins into individual viral proteins is an important step of gene expression, and viral proteases that perform this step have been targeted to develop antiviral drugs. Following entry of the flavivirus, HCV, its RNA genome is translated into a single very large (~3000 residue) polypeptide. Subsequent proteolytic processing of the polypeptide to release functional proteins is accomplished through a combination of host cell proteases and two virally encoded proteases—nonstructural protein 2 (NS2) and NS3. Drug discovery efforts have focused on NS3, a serine protease that associates with a second viral protein (NS4a), which both increases its

enzymatic activity and anchors the protein to the membrane to augment processing efficiency.[237] NS3 autoproteolytically cleaves between NS3 and NS4A and then cleaves to generate NS4A (its cofactor), NS4B, NS5A, and NS5B.[310] NS3/4a may also play a role in blunting the host immune response by cleaving cellular substrates involved in double-stranded RNA sensing and induction of the type 1 interferon response.[256,362]

Prior to 2011, the standard of care for HCV infection was treatment with a combination of interferon-α (IFN-α) and ribavirin (a nucleoside analog; see below), which had limited efficacy that varied across the 6 HCV genotypes and had major side effects. The development of NS3/4A inhibitors drugs serves as a case study for how medical need drives medicinal chemistry aided by structural biology.

As a serine protease, NS3 employs the typical serine, histidine, aspartate "catalytic triad" to effect peptide cleavage.[101] Like most proteases, NS3 also has a pocket called the oxyanion hole that stabilizes the negative charge that results following substrate cleavage. All of the approved HCV NS3/4A inhibitors have extensive binding contacts with the catalytic triad both to optimize binding affinity and to ensure that drug resistance mutations that affect these contacts would result in a fitness penalty. Additional interactions also occur with 3-4 substrate recognition sites on the amino terminal side of the bond cleavage site (termed P1-P4 for the inhibitor (see Fig. 14.6A for an example, telaprevir)).

Boceprevir and telaprevir were the first 2 approved direct-acting antivirals for treatment of HCV.[148] They are linear peptidomimetics interacting with the S1-S4 pocket of the protease (Fig. 14.6A and B). Both employ an alpha ketoamide to covalently, but reversibly, engage the catalytic serine. Additional, critical binding interactions include engagement of His 57 of the catalytic triad and residues within the peptide binding site, and hydrogen bonding contacts at positions 155 and 156 (Fig. 14.6C). The compounds possess low nM potency against the genotype 1 NS3/4A enzyme and low triple digit nM potency in cell-based assessments employing subgenomic replicon systems.[191,259,346,450] These molecules represented a significant advance in HCV therapy, substantially increasing the rate of sustained virologic response (cure) and decreasing the treatment duration. However, the first-generation inhibitors were still combined with IFN-α and ribavirin, adding the molecules' side effects to those of IFN-α and ribavirin; their modest potency and suboptimal pharmacokinetics (PK) required three times a day dosing; and resistance mutations developed at six different positions.[216,379,388,416,423] Crucially, they were less potent against nongenotype 1 HCV.[96,158,404]

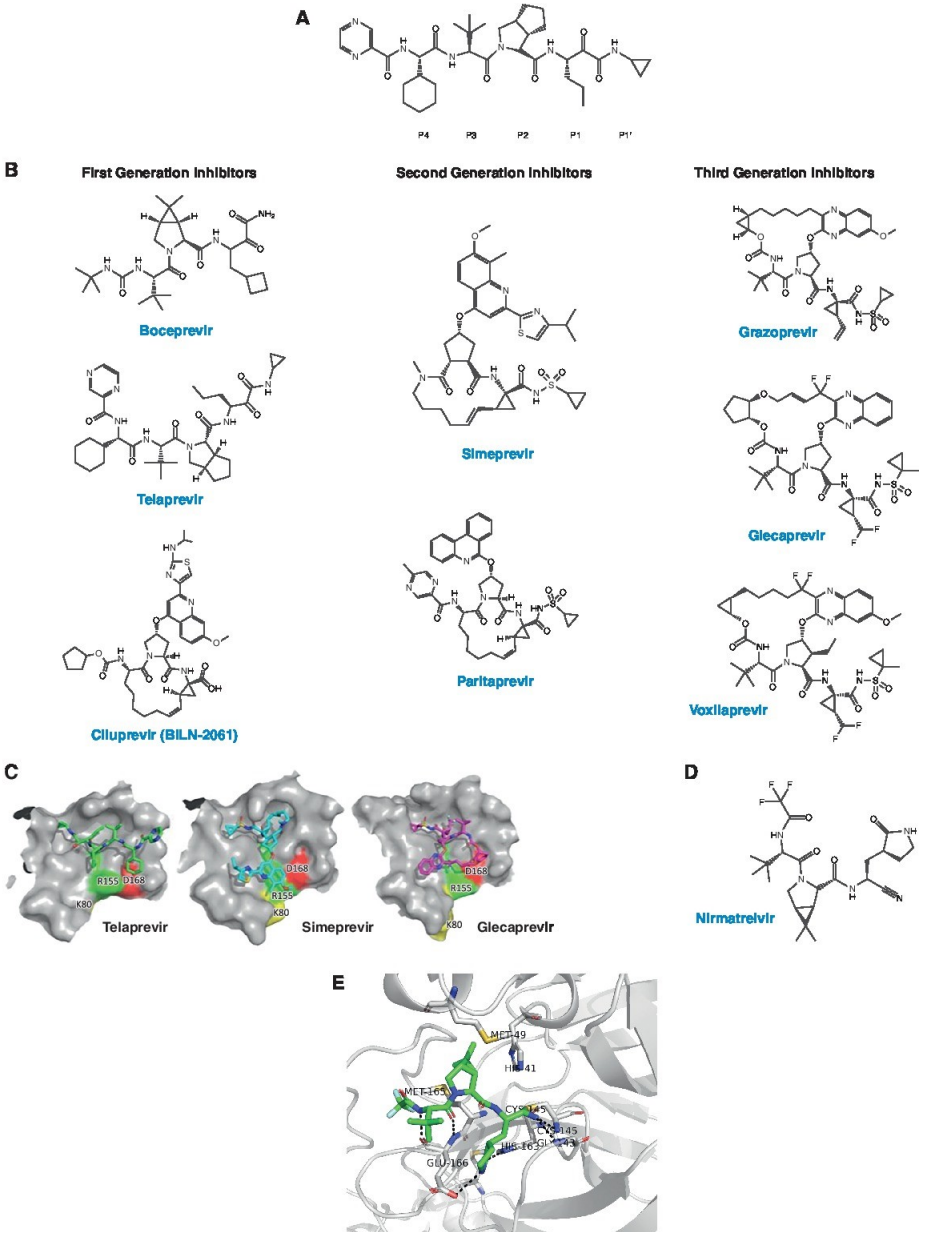

FIGURE 14.6 **Protease inhibitor antiviral drugs that block polyprotein processing of HCV and SARS-CoV2**. **A**. Telaprevir is arranged as a linear peptidomimetic, with moieties P1-P4 and P1' that interact with the corresponding pockets of the HCV NS3/4A protease. **B**. Structures of first-generation approved inhibitors telaprevir and boceprevir along with the investigational drug ciluprevir are shown in the left column; second-generation (simeprevir and paritaprevir) and third-generation (grazoprevir, glecaprevir, voxilaprevir) inhibitors (middle and right column, respectively) were based on ciluprevir. See text for details. **C**. The binding of an example of each generation of inhibitor (telaprevir, simeprevir, glecaprevir) to the HCV NS3/NS4 protease is shown. The positions of amino acids K80, R155, and D168, which bind to some inhibitors, and not others are shown. See text for details. **D**. The structure of the SARS-CoV-2 main protease inhibitor, nirmatrelvir. **E**. Schematic of the binding of nirmatrelvir in the main protease active site. Nirmatrelvir forms a reversible covalent bond via the P1' nitrile with Cys145 as well as hydrogen bonds with the backbone NH of Cys145, Gly143, His163, and Glu166, and hydrophobic interactions with a pocket formed by Met49, His41, and Met165. Panels C and E were kindly provided by Paul Charifson.

Most second-generation inhibitor efforts were based on ciluprevir (also known as BILN-2061), a macrocyclic compound that did not progress to approval but was the first HCV protease inhibitor to demonstrate antiviral activity in humans[236] (Fig. 14.6B). Incorporation of a macrocyclic structure improved compound potency, cellular permeability, and bioavailability.[398] A common modification of ciluprevir that was broadly employed was replacement of the P1 carboxylic acid with a more druglike ketosulfonamide[19,218,376] (Fig. 14.6B). The substitution retains extensive contacts within the oxyanion hole, and like the analogous carboxylate of ciluprevir does not form a covalent bond with the catalytic serine. Similar to ciluprevir, the second-generation inhibitors contain a 14-16 membered macrocyclic ring linking P1 and P3. A third common structural feature was incorporation of a large hydrophobic substituent at P2, which improved target binding through interaction with hydrophobic shelf in that position[293,380] (Fig. 14.6C). Two inhibitors from this structural class, simeprevir and paritaprevir (Fig. 14.6B), progressed to U.S. FDA approval as components of some of the first all oral therapies for HCV, eliminating the use of IFN-α. Both molecules were substantially more potent, were dosed once a day, and the combination therapies were highly effective against genotype 1 within 12-24 weeks.[128,245] However, three of the mutations identified with the first-generation inhibitors, especially those affecting NS3 residues 155 and 168, still conferred resistance to the new drugs, and one of these is a signature polymorphism of genotype 3 (D168Q).[293,451]

The critical change in molecular design of third-generation inhibitors was moving the attachment point for the macrocycle from P1 to position 3 of the P2 proline (Fig. 14.6B).[167,170,434] This allowed decreased interaction with positions 155 and 168 (Fig. 14.6C) and dramatic improvements in potency against genotype 3 and mutants resistant to the first 2 generations of NS3/4A inhibitors.[169,319] A second common design feature was incorporation of a quinoxaline within the macrocyclic ring system (Fig. 14.6B). This resulted in a more extensive pi stacking interaction with His 57 of the catalytic triad, which was critical in improving genotype 3 potency[434] (Fig. 14.6C). These changes provided molecules with sub nM potencies when assessed in subgenomic replicons across the six HCV genotypes, as well as drug resistance mutations identified in the first 2 generations of molecules. Glecaprevir and voxilaprevir (Fig. 14.6B and C) are components of two all oral, multidrug regimens approved for treatment of all HCV genotypes.

Inhibition of Polyprotein Processing: A SARS-CoV-2 Mpro Inhibitor

Following entry of the coronavirus, SARS-CoV-2, its ~30 kilobase viral RNA genome is expressed as 2 large polyproteins, pp1a and pp1ab encoding 11 and 16 viral proteins, respectively.[446] The polyproteins are processed by two essential, virus-encoded cysteine proteases, the papainlike protease (PLpro) and the chymotrypsinlike or main protease (3Clpro or Mpro).[446] Nirmatrelvir (Fig. 14.6D) is a covalent reversible tetrapeptide peptidomimetic inhibitor of the SARS-CoV-2 Mpro. The chemical structure is related to that of the HCV NS3/4A inhibitor, boceprevir (Fig. 14.6B), which was found to inhibit SARS-CoV-2 Mpro.[23,138,274] Optimization yielded nirmatrelvir, which has a 3 nM K_i for inhibition of the SARS-CoV-2 Mpro.[335] The compound forms a reversible covalent bond with the catalytic cysteine (Cys 145) (Fig. 14.6E), but otherwise does not make extensive contact with the oxyanion hole. The P1 cycloglutamine forms a bidentate hydrogen bond to histidine 163 and the carboxylic acid of glutamate 166, while the bridged proline at P2 binds a hydrophobic pocket formed by Met49, His41, and Met165 (Fig. 14.6E).[335] Nirmatrelvir is highly selective for Mpro with no significant binding noted to a broad panel of mammalian proteases, receptors, and enzymes.[335]

Nirmatrelvir exhibits ~100 nM EC_{90} values in various cells infected with SARS-CoV2.[335] Preclinical *in vivo* efficacy was demonstrated in a mouse adapted SARS-CoV-2 infection model dosing 4 hours post infection where nirmatrelvir treatment demonstrated a significant decrease in lung viral titer and virus-associated histopathologic findings.[335] However, nirmatrelvir is poorly bioavailable across various species including humans, prompting coadministration with the HIV protease inhibitor, ritonavir (see under *Inhibitors of Viral Assembly/Egress*), which also inhibits the liver enzyme, Cyp 3A4, that metabolizes nirmatrelvir.[335]

Inhibition of Viral Genome Replication

The largest class of antiviral drugs comprises those that inhibit viral genome replication, most commonly by inhibiting a viral polymerase. Viruses whose genome replication has been successfully targeted to yield FDA-approved or -authorized drugs include the herpesviruses HSV, HCMV, and varicella zoster virus (VZV), the poxvirus variola virus (VV), the lentivirus HIV, the hepadnavirus HBV, the flavivirus HCV, and the coronavirus SARS-CoV-2. Most of the approved drugs are nucleoside analogs that inhibit viral polymerases (Fig. 14.7). Some of these nucleoside analogs mimic nucleoside monophosphates, so they may be considered nucleotide analogs. One nucleoside analog doesn't inhibit a polymerase, but rather a protein kinase. Other inhibitors of genome replication are nonnucleoside inhibitors of a polymerase, inhibitors of a different replication protein of HCV, and inhibitors of HIV integration.

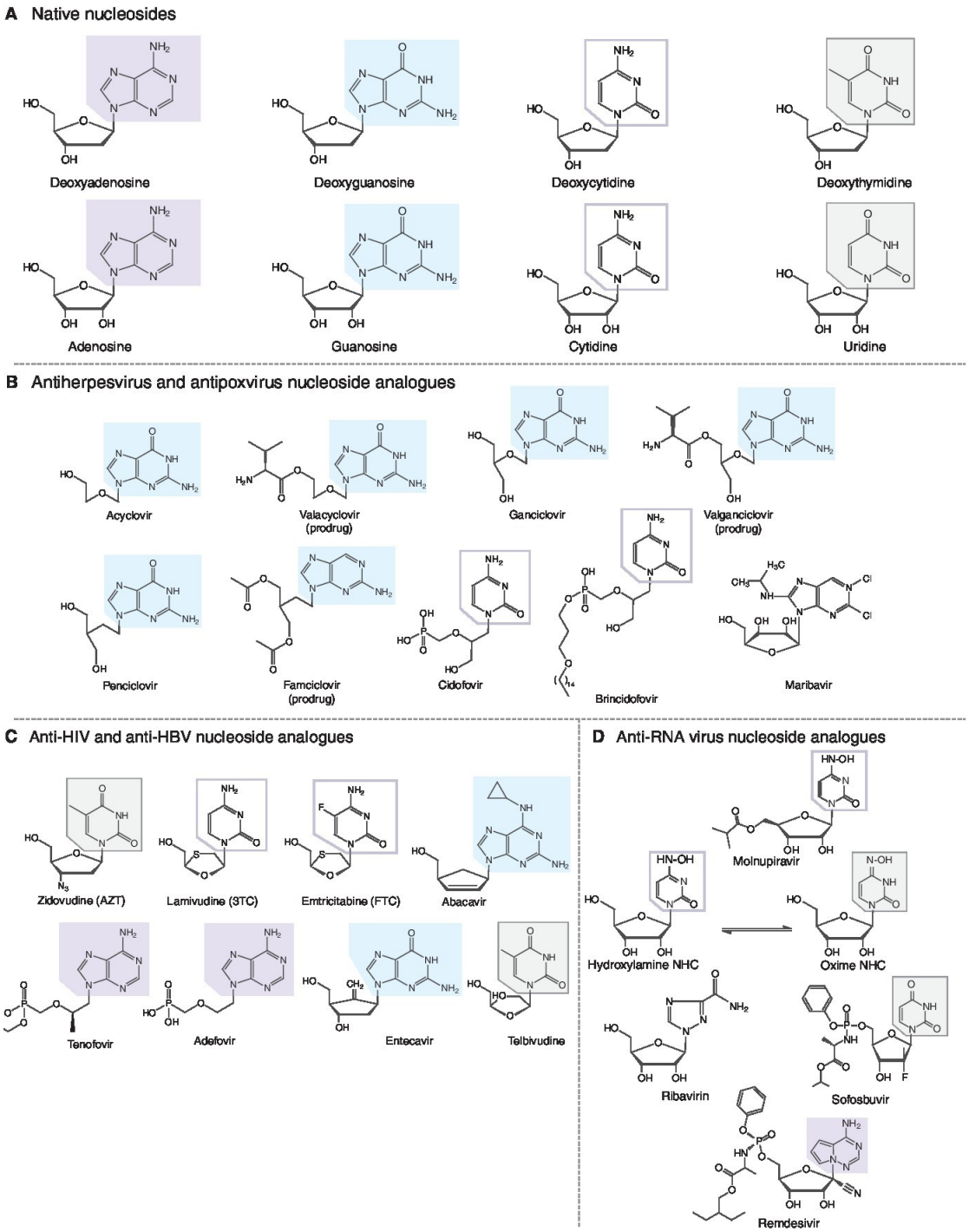

FIGURE 14.7 **Structures of antiviral nucleoside analogs**. **A**. The deoxynucleosides (*top row*) used as precursors for DNA synthesis and the ribonucleosides (*second row*) used as precursors for RNA synthesis are depicted in their *anti* configurations. The base moieties are shaded. **B**. Structures of antiherpesvirus and antipoxvirus nucleoside analogs mimic the deoxynucleoside, deoxyguanosine, except for cidofovir, which mimics the deoxynucleotide dCMP, and maribavir, which contains a purine analog joined to an L-ribose. All but maribavir contain acyclic moieties that mimic deoxyribose. Phosphorylation of all but maribavir results in inhibitors of viral DNA polymerase; unphosphorylated maribavir inhibits the HCMV protein kinase, UL97. Valacyclovir, valganciclovir, famciclovir, and brincidofovir are prodrugs of ACV, ganciclovir, penciclovir, and cidofovir, respectively. **C**. Anti-HIV and anti-HBV nucleoside analogs are obligate chain terminators except the anti-HBV drugs entecavir and telbivudine. The drugs in the top row plus tenofovir are approved for use against HIV. The drugs in the bottom row plus lamivudine are approved for use against HBV. Zidovudine and telbivudine mimic dT, lamivudine and emtricitabine mimic dC, tenofovir and adefovir mimic dAMP, and abacavir and entecavir mimic dG. Lamivudine, emtricitabine, and telbivudine are l-stereoisomers, while the other nucleoside analogs are d-stereoisomers. **D**. Anti-RNA virus nucleoside analogs include molnupiravir, which is a cytidine analog prodrug that is converted to β-D-N4-hydroxycytidine (NHC), which can exist in two tautomeric forms, an oxime and a hydroxylamine, and has been authorized for use against SARS-CoV-2. NHC-triphosphate is not a chain terminator. Other anti-RNA virus nucleoside analogs include ribavirin, which contains a purine mimic attached to ribose, and is approved for use against the RNA viruses HCV and respiratory syncytial virus; sofosbuvir, which is a cytidine monophosphate analog prodrug containing a 2′ fluoro group in place of the usual hydroxyl and is approved for use against HCV; and remdesivir, which is an adenosine monophosphate analog prodrug containing an azido-modified ribose active against SARS-CoV-2 and has been authorized for use against SARS-CoV-2. (Modified with permission from Li JZ, Coen DM. Pharmacology of viral infections. In: Golan DE, Armstrong EJ,

Nucleoside Analogs Targeting Polymerases: General Considerations and Specific Examples

Nucleoside analogs that clearly target viral polymerases are approved or authorized for treatment of certain herpesviruses, VV, HBV, HIV, HCV, and SARS-CoV-2. These drugs must be activated by phosphorylation to mimic nucleoside triphosphates to exert their effect. Host enzymes are generally responsible for phosphorylation of nucleoside analogs, with the exception of most nucleoside analogs active against herpesviruses where a viral kinase is responsible for the first phosphorylation. Selectivity of nucleoside analogs is largely dictated by the relative efficiency with which the nucleoside analog triphosphate can inhibit viral replication vs cellular functions.

Phosphorylated nucleoside analogs can act on viral polymerases in multiple ways. All compete with binding of the natural substrate (deoxynucleoside triphosphate (dNTP) for DNA polymerase, ribonucleoside triphosphate (NTP) for RNA polymerase) to the polymerase, and all are also incorporated into the growing nucleic acid chain. Either or both of these features—inhibition and incorporation—can be important for antiviral activity. Incorporation of the analog can result in three outcomes: (1) obligate chain termination where the analog is unable to be extended by the polymerase because it lacks a 3′ hydroxyl (OH) group; (2) nonobligate chain termination where the analog retains a 3′ hydroxyl group yet DNA synthesis is terminated, sometimes following incorporation of one or more additional nucleotides (delayed termination); and (3) relatively unimpeded elongation.

These and other principles of nucleoside analogs are illustrated for each kind of outcome by two older drugs, acyclovir and ganciclovir, and a newer drug, molnupiravir.

Acyclovir, an Obligate Chain Terminator Phosphorylated by a Viral Kinase

Acyclovir (ACV) and its more orally available valine ester, valacyclovir (Fig. 14.7B), are approved for treatment of diseases caused by HSV and VZV. ACV consists of a guanine base attached to an acyclic sugarlike molecule that lacks the 2′ and 3′ moieties found in deoxynucleosides (Fig. 14.7B). The mechanism of ACV action[114] is presented in Figure 14.8. The TK encoded by HSV and VZV phosphorylates ACV (Fig. 14.8A). Crystal structures of HSV TK in complex with thymidine or ACV[48,58,469] show that the guanine moiety of ACV interacts with the same residues as does the thymine moiety of thymidine. The HSV or VZV TKs phosphorylate ACV more efficiently than host kinases leading to 30- to 100-fold more phosphorylated ACV in infected cells, which accounts for much of ACV's antiviral selectivity.

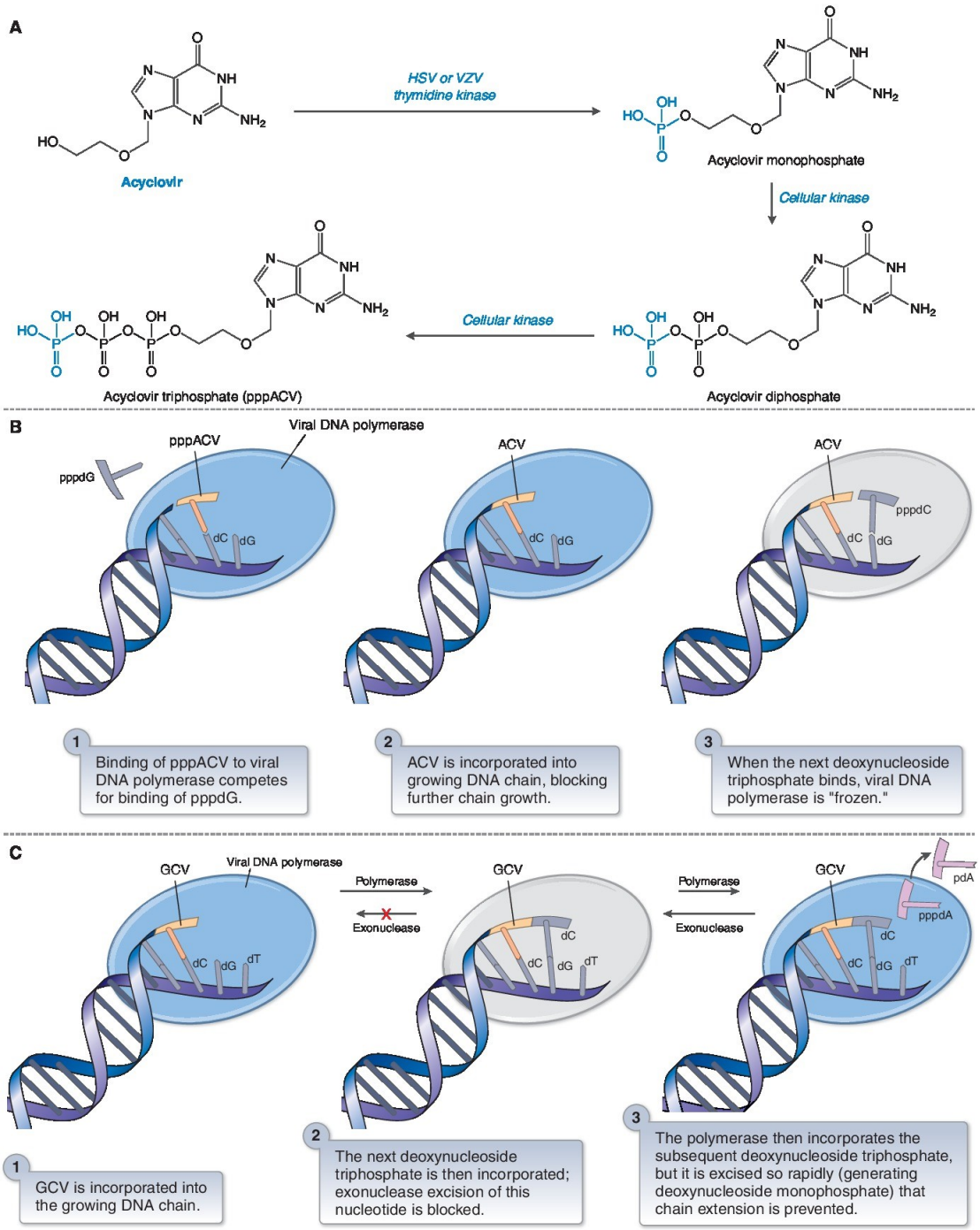

FIGURE 14.8 **Mechanisms of action of ACV and GCV**. **A**. ACV is selectively phosphorylated by herpes simplex virus (HSV) or varicella zoster virus (VZV) thymidine kinase to generate ACV monophosphate. Host cellular enzymes then sequentially phosphorylate the drug monophosphate to the diphosphate and triphosphate (pppACV) forms. **B**. ACV triphosphate has a three step mechanism of inhibition of herpesvirus DNA polymerase *in vitro*: (1) the drug triphosphate acts as a competitive inhibitor of dGTP (pppdG) binding; (2) the drug triphosphate acts as a substrate and is incorporated into the growing DNA chain across from dC in the template, terminating elongation; and (3) the polymerase becomes trapped on the ACV-terminated DNA chain when the dNTP binds (here shown as pppdC, dCTP, which would be templated by dG). **C**. Ganciclovir triphosphate (GCV-TP) is a nonobligate chain terminator. In the model for its multistep mechanism of action, GCV-TP is incorporated into the growing DNA chain across from dC in the template (*forward arrow*) more efficiently than it is removed by the polymerase's exonuclease activity. The next deoxynucleoside triphosphate (dNTP) is incorporated across from its templating base; the exonuclease is unable to remove this incorporated nucleotide (*reverse arrow* with an x through it). When the subsequent nucleotide is incorporated, it is rapidly removed by the exonuclease, generating dNMP in a process known as idling, and preventing further chain extension. (Modified with permission from Li JZ, Coen DM. Pharmacology of viral infections. In: Golan DE, Armstrong EJ, Armstrong AW, eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. 4th ed. Wolters Kluwer; 2017:694-722.)

Phosphorylation of ACV by the viral TK produces the compound ACV-monophosphate, which is converted sequentially to ACV-diphosphate and ACV-triphosphate (ACV-TP) by cellular enzymes[305,306] (Fig. 14.8A). ACV-TP then inhibits HSV and VZV DNA polymerases. Inhibition of HSV DNA polymerase *in vitro* is a three-step process (Ref.[361]; Fig. 14.8B). In the first step, ACV-TP competitively inhibits dGTP incorporation. Next, ACV-TP acts as a substrate and is incorporated into the growing DNA chain opposite a C residue. Finally, the polymerase translocates to the next

position on the template but cannot add the next dNTP because ACV contains no 3′-hydroxyl; hence, ACV-TP is an obligate chain terminator. Provided that the next dNTP is present, the viral polymerase freezes at this final step in a "dead-end complex," leading to apparent inactivation of the enzyme. This mechanism appears to be common among obligate chain terminators and may be due to the polymerase becoming locked in its closed conformation induced by binding to dNTP and primer template, in this case terminated by ACV. There is biochemical and genetic evidence for selectivity at each of these steps.[193,202,285,360]

There is considerable variety in ACV-resistant mutants. ACV selects mainly for *tk* mutations[83,387,425] that ablate (TK-negative) or reduce (TK-partial) TK activity, or alter TK so that it fails to phosphorylate ACV but continues to phosphorylate dT (TK-altered). ACV-resistance mutations can also alter the viral polymerase catalytic subunit (Pol) to be less inhibited by the drug (reviewed in[239]). Structural studies have provided details on how *tk* and *pol* mutations affect binding and catalysis of ACV and ACV-TP, respectively, and suggest further that *pol* mutations can confer resistance by disfavoring the closed complex[48,58,134,193,269,469](Protein Data Bank ID: 7M5R).

Nearly all ACV-resistant HSV mutants exhibit some degree of attenuation in assays of pathogenesis in animal models, with TK-negative mutants being the most attenuated and TK-partial and certain *pol* mutant being the least (reviewed in Refs.[82,239]). Interestingly, certain nonsense and frameshift mutations that might be expected to inactivate TK do not do so, due to translational mechanisms that permit low levels of TK that suffice for aspects of pathogenesis.[160,198,341] Additionally, mixtures of drug-resistant mutants with each other or with drug-sensitive virus can complement each other for drug resistance and pathogenicity.[82,239] Thus, it is important to consider heterogeneity as a factor influencing drug resistance and pathogenicity.

Ganciclovir: A Nonobligate Chain Terminator Phosphorylated by a Viral Kinase

A congener of ACV, ganciclovir (Fig. 14.7B), was the first drug approved for use against HCMV. Ganciclovir (GCV) is now also produced as the more orally available valine ester, valganciclovir, with the same valyl modification as valacyclovir (Fig. 14.7B). GCV, unlike ACV, has the equivalent of a 3′-OH moiety (Fig. 14.7B). Much as viral TK phosphorylates ACV in HSV- and VZV-infected cells, an unusual viral protein kinase, UL97, phosphorylates GCV in HCMV-infected cells.[181,265,301,417,431] Cellular kinases can convert GCV monophosphate to GCV triphosphate.

Like ACV triphosphate, GCV triphosphate is both a selective competitive inhibitor and substrate for viral DNA polymerase.[38,151,282,285,363,410] However, due to its equivalent of a 3′-OH, GCV triphosphate is not an obligate chain terminator. Nevertheless, after incorporating GCV, HCMV DNA polymerase terminates synthesis after incorporating one additional nucleotide.[363] Such termination following incorporation of subsequent nucleotides is called delayed termination. For GCV, this requires the 3′-5′ exonuclease (Exo) activity of viral DNA polymerase.[62] A model for nonobligate chain termination by GCV is shown in Figure 14.8C: HCMV DNA polymerase incorporates GCV readily, and then the next dNTP. Of note, once that dNTP is incorporated, even the Exo of wild-type enzyme is unable to excise it. Wild-type enzyme can then add the subsequent dNTP to this primer template, but only slowly, and the Exo removes it before it can be used for further extension, a process known as idling[62,63] (Fig. 14.8C), explaining delayed nonobligate chain termination. Loss of Exo activity eliminates idling, permitting extension, thereby overcoming chain termination and conferring GCV resistance, which might require DNA repair, too.[62] Consistent with this model, a different mutant polymerase extends GCV-containing primers more rapidly to overcome chain termination.[63] Several other nucleoside analogs are nonobligate chain terminators; where known, their mechanisms of termination will be discussed (see below).

Biochemical and genetic studies indicate that selectivity of GCV at the phosphorylation step and the DNA polymerase inhibition step for HCMV are not as great as the selectivity of ACV against those steps for HSV. Accordingly, the drug is more toxic than ACV. Toxicity is most commonly manifested in patients as bone marrow suppression, especially neutropenia.[322]

Most GCV-resistant mutants contain *UL97* mutations, but these have a limited distribution in the gene,[24,153] suggesting that they alter substrate specificity. Unlike HSV TK, HCMV UL97 protein kinase is very important for viral replication in cultured cells[355]; see discussion of maribavir below. Numerous different *pol* mutations have been shown to confer ganciclovir resistance (reviewed in Refs.[24,153]). As noted above, some lie in regions important for Exo activity, while other lie in regions important for DNA polymerase activity. As these mutants cause disease in humans, they clearly retain fitness.

Molnupiravir: A Nonchain Terminating Analog Phosphorylated by Host Kinases

Molnupiravir is a 5′ isopropyl ester prodrug of β-D-N4-hydroxycytidine (NHC, Fig. 14.7D) that is authorized for the treatment of SARS-CoV-2. Upon oral administration, molnupiravir is rapidly converted to NHC by hydrolysis of the isopropyl moiety, and after cellular uptake, NHC is phosphorylated to the nucleoside triphosphate by host kinases (reviewed in Ref.[338]). NHC has antiviral activity against SARS-CoV-2 and multiple other coronaviruses in cell culture and in animal models, with submicromolar EC_{50} values.[91,397,454]

NHC can exist in two tautomeric forms (Fig. 14.7D), allowing it to pair with either G or A. *In vitro* biochemical studies with the RdRp from SARS-CoV-2 demonstrate that NHC-TP competes most effectively with CTP when incorporated in a growing RNA strand opposite a template G, although NHC-TP can also be incorporated opposite a template A. Unlike the triphosphates of ACV or GCV, NHC-triphosphate does not cause chain termination.[155,209,279,299] Efficient incorporation and extension may allow NHC to avoid excision via the coronavirus proofreading exonuclease, which associates with the RdRp.[5]

Several lines of evidence suggest incorporation of NHC results in full-length, but less functional viral RNAs. Studies with a rodent coronavirus (murine hepatitis virus, MHV) and SARS-CoV-2 suggest that NHC has substantially larger effects on infectivity of supernatant virus than on total or supernatant viral RNA.[5,397] Studies of molnupiravir in SARS-CoV-2–infected patients are also consistent with greater effects on infectivity than on production of RNA.[137] The most popular hypothesis for how this occurs is "error catastrophe," wherein NHC incorporation causes an accumulation of mutations within viral RNAs until the resulting virus is not viable.[397] This mechanism was first proposed for another ribonucleoside analog, ribavirin (see below). For molnupiravir, not only can NHC be incorporated opposite both G and A as noted above, but both G and A can form base pairs opposite NHC in the template, resulting in mutagenesis even more efficiently than when NHC is incorporated opposite A.[155,209,279,299] Studies in cells infected with SARS-CoV-2 demonstrate that NHC causes an increase in error frequency with a preference for all four transitions,[397] which implies that NHC is incorporated into both positive and negative strands during genome replication.

Increased frequencies of transition mutations have also been observed in SARS-CoV-2–infected people treated with molnupiravir.[137]

Error catastrophe may not account for all of the antiviral activity of molnupiravir. For example, in an MHV study,[5] G:A and C:U transition frequencies increased only ~40% at concentrations that decreased infectivity 100 000-fold, whereas in other MHV studies, a mutation that inactivates a proofreading exonuclease was reported to increase mutation frequencies 1500%, yet decreased peak viral yields only ~10-fold.[113,159] Presently, as transition error frequency only serves as an indirect measure of the degree of incorporation of NHC into viral genomic and mRNA, it cannot be excluded that insertion of NHC into viral mRNA could result in mistranslation, alter ribosomal frameshifting, and/or impair posttranscriptional modifications important for RNA stability or translation, in which case molnupiravir might, at least in part, inhibit viral gene expression. An additional possibility is that NHC or its metabolites affect other viral functions. Regardless, molnupiravir is the best example of a nucleoside analog that acts against the virus for which it is used via a viral polymerase yet is not a chain terminator.

NHC appears to have a high barrier to resistance in that coronavirus variants with only ~twofold decreases in susceptibility have been isolated, and changes in drug sensitivity have not been correlated with any signature mutations.[5] NHC retains activity against mutants resistant to remdesivir (see below), another nucleoside analog active against SARS-CoV-2.[397] At this writing, there is little information regarding resistance in treated patients.

Antiherpesvirus Nucleoside Analogs Other Than ACV and GCV and an Antipoxvirus Nucleoside Analog

Aside from ACV and GCV, a number of antiviral nucleoside analogs have been developed against herpesviruses. These include vidarabine, idoxuridine, and trifluridine, which have been primarily used as topical treatments for ocular HSV infections. These drugs have been superseded by more selective compounds and/or have poorly understood mechanisms and will not be discussed further.

Penciclovir and Famciclovir

Penciclovir (Fig. 14.7B) is approved for topical use for cold sores caused by HSV, and its orally available prodrug, famciclovir, which is a diacetyl ester of 6-deoxypenciclovir (Fig. 14.7B), is approved for use against cold sores, genital herpes, and shingles (caused by VZV). Penciclovir's mechanism of action is overall similar to that of ACV, but penciclovir triphosphate is a nonobligate chain terminator of DNA synthesis, due to its 3' hydroxyl-like moiety similar to that of GCV, although it does permit at least some extension of primers following incorporation.[111] Penciclovir-resistant mutants are similar to ACV-resistant mutants with some subtle differences.[68,387]

Cidofovir and Brincidofovir

Cidofovir, a phosphonate-containing acyclic cytosine analog, is approved for use against HCMV retinitis. With its phosphonate group, it is, in effect, a nucleotide analog that mimics dCMP (Fig. 14.6). This charged moiety likely accounts for the relatively poor uptake of cidofovir into cells[188] and poor oral bioavailability. Once inside the cell, cidofovir is metabolized via cellular enzymes to its monophosphate (akin to a diphosphate) and diphosphate (akin to a triphosphate), and to a third phosphorylated form that contains a choline adduct.[76,188] These phosphorylated forms have very long intracellular half-lives, which may be due in part to the choline metabolite serving as a reservoir.[2,188] This property contributes to the prolonged antiviral activity of cidofovir,[309] which provides the therapeutic advantage of infrequent dosing. Also, because cidofovir uses cellular kinases for its phosphorylation, it is active against *UL97* mutants that are resistant to ganciclovir,[272] although this also removes the selectivity gained by requiring a viral kinase for efficient phosphorylation.

The diphosphorylated form of cidofovir is an analog of dCTP that inhibits HCMV DNA polymerase more potently than it does various cellular DNA polymerases.[67,188,480] It is a nonobligate chain terminator, which when incorporated into DNA, slows elongation, and, when two cidofovir residues are incorporated in a row,[479,480] causes chain termination. Many of the *pol* mutations that confer resistance to ganciclovir confer resistance to cidofovir, suggesting similar mechanisms of chain termination due to exonuclease-mediated idling[62,63] and similar fitness.

Efforts to overcome the lack of oral availability and nephrotoxicity of cidofovir led to the development of a hexadecycloxypropyl ester, conjugated to the phosphonate, brincidofovir (Fig. 14.7B). This not only increased oral availability but also cell uptake and conversion to the diphosphate.[190] Brincidofovir shows activity in cell culture against many double-stranded DNA viruses that encode their own DNA polymerases, including submicromolar potency against orthopoxviruses, including variola virus (reviewed in Ref.[133]), which is the cause of smallpox. Clinical trials of brincidofovir for HCMV and adenoviruses did not succeed, but those studies and others provided safety information in humans, while animal studies of the drug's ability to reduce mortality from various orthopoxviruses in animal models provided efficacy data for treatment of smallpox, for which human trials are not deemed practical or ethical (reviewed in Ref.[133]). Related orthopoxviruses readily develop resistance to brincidofovir due to alterations in DNA polymerase, but thus far, those mutants show reduced virulence in animal models.[9,109] Still, the recovery of cidofovir-resistant HCMV mutants from patients suggests that fit, disease-causing brincidofovir-resistant smallpox viruses could arise. The FDA approved brincidofovir for treatment of smallpox in 2021.

A Nucleoside Analog That Inhibits HCMV Protein Kinase to Impair Genome Replication

As discussed above in the section on ganciclovir, HCMV encodes an unusual protein kinase, UL97. This enzyme, although not strictly essential for production of infectious HCMV, is very important, especially in nondividing cells where it plays a role in viral DNA synthesis.[37,211,355,364,473] Maribavir is the L-stereoisomer of a benzimidazole riboside nucleoside analog (Fig. 14.7B), which was found to inhibit HCMV replication and viral DNA synthesis at submicromolar concentrations.[37] Its toxicity in animal models, and subsequently in human clinical trials, was found to be less severe than that of older HCMV drugs, and it is orally available.[225,471] Maribavir inhibits UL97 kinase activity with low nM IC_{50}, and *UL97* mutations were shown to confer maribavir resistance.[37] The locations of resistance mutations and maribavir's resemblance to a nucleoside suggest the hypothesis that the drug binds at or near the ATP-binding pocket of UL97.[73] Consistent with this hypothesis, maribavir has been reported to inhibit UL97 autophosphorylation competitively with ATP.[395] However, another study reported that maribavir inhibits competitively with a nucleoside analog substrate for phosphorylation.[150] Resolution of these seemingly

discordant results may require structural analyses.

How does maribavir inhibit HCMV DNA synthesis? Although UL97 can phosphorylate a subunit of HCMV DNA polymerase, UL44, substitution of UL44's sites of phosphorylation by UL97 with alanine had no more than modest effects on viral replication.[403] On the other hand, UL97 mimics host cyclin-dependent kinases to phosphorylate retinoblastoma protein (Rb), which controls transcription of genes for host proteins important for cellular DNA synthesis.[194,211,356] Expression of a papillomavirus oncoprotein that leads to degradation of Rb family proteins compensates for the loss of UL97 for viral DNA synthesis in nondividing cells, while a mutant form of this protein that is specifically disrupted for Rb degradation does not.[211] These results and the lack of HCMV-encoded enzymes that increase nucleotide pools such as ribonucleotide reductase and TK support a hypothesis where HCMV UL97 inactivates Rb family members to activate corresponding host enzymes to increase dNTP pools. Although there is a publication that argues against this hypothesis[231] and although no direct measurements of the effects of UL97 on nucleotide pools have been reported, this remains an appealing hypothesis to explain how maribavir inhibits DNA synthesis. Nevertheless, which Rb family–controlled proteins are crucial for HCMV DNA synthesis remain unclear, and it remains possible that phosphorylation of other substrates of UL97 also contribute to its role in viral DNA synthesis. Notably, loss of UL97 activity has little effect on HCMV DNA synthesis in dividing cells; in that setting, UL97 appears to be particularly important for viral nuclear egress (see under *Inhibitors of Viral Assembly/Egress*).[211,364]

In HCMV-infected cells in culture, resistance to maribavir can arise not only from *UL97* mutations but also from mutations in the *UL27* gene.[223] Loss of *UL27* function results in increased cyclin-dependent kinase activity, which appears to account for the modest resistance observed.[365] Maribavir was approved by the FDA in 2021 to treat patients with posttransplant HCMV disease that is refractory to treatment with ganciclovir, valganciclovir, foscarnet, or cidofovir. Maribavir resistance due to *UL97* mutations arose in ~20% of treated patients in the clinical trials and was associated with treatment failures; *UL27* mutations were less common and their clinical relevance remains unclear.[74]

Anti-HIV and HBV Nucleoside Analogs

As discussed elsewhere in this edition, the lentivirus, HIV, and the hepadnavirus, hepatitis B virus (HBV) replicate their genomes through DNA and RNA intermediates, respectively, using a DNA polymerase that can copy RNA. The HIV reverse transcriptase (RT) and the HBV DNA polymerase are thus essential for viral replication and differ substantially from cellular polymerases, making them excellent targets for antivirals. The success of antiherpesvirus nucleoside analogs provided a rationale for the development of nucleoside analogs active against HIV and subsequently HBV.

Numerous nucleoside analogs have been approved to treat HIV (Fig. 14.7C), all of which are obligate chain terminators. Thus, many of the principles that govern their mechanisms of action and resistance are similar to those of ACV, although there are important differences. For the sake of brevity, this chapter will dwell only on the anti-HIV nucleoside analogs, AZT, and two drugs closely related to each other, lamivudine and emtricitabine, to illustrate principles of this class of antivirals.

Zidovudine

Zidovudine, (3′-azidothymidine, AZT), which was first reported to have anti-HIV activity in 1985,[307] is a thymine base attached to a sugar in which the normal 3′ hydroxyl has been replaced by an azido group (Fig. 14.7C). Although rarely used now in wealthy countries, zidovudine (3′-azidothymidine, AZT) is still on the WHO essential medicines list. AZT is an excellent substrate for cellular TK, which phosphorylates ZDV to its monophosphate (AZT-MP).[140] The monophosphate is then converted to the diphosphate form by cellular thymidylate kinase and to the triphosphate form (AZT-TP) by cellular nucleoside diphosphate kinase.[140] The activity of AZT and other anti-HIV nucleoside analogs can vary depending on the activity of cellular kinases in the infected cell. For example, activated lymphocytes upregulate thymidine kinase, which is needed for DNA replication and cellular proliferation; thymidine analogs are thus more potent in activated than in quiescent CD4 lymphocytes.[399]

AZT-TP is a substantially more potent inhibitor of and substrate for HIV RT than of the human DNA polymerases that have been tested.[66,140,411] This biochemical selectivity is reflected in the degree of resistance of AZT-resistant mutants, which can be >100-fold in some cell types.[240] Nevertheless, its toxicity in particular cells is a serious clinical issue. In particular, AZT causes bone marrow suppression that manifests most commonly as neutropenia and anemia.[370] AZT toxicity appears to be due not only to the effects of AZT-TP on cellular polymerases but also to the effects of AZT-monophosphate, which is both a substrate for cellular thymidylate kinase and an inhibitor of this essential enzyme.[140] For a number of other anti-HIV nucleoside analogs, a key determinant of toxicity appears to be the mitochondrial DNA polymerase (DNA polymerase γ[61,249,285]).

AZT-resistance mutations accumulate in the HIV *pol* gene that encodes RT[43,77,212,242] (Fig. 14.9). Interestingly, four or more mutations are required to confer high-level resistance, but some of the mutations confer little or no resistance on their own.[212,241,242] These latter mutations appear to be selected because they boost resistance by the other mutations or compensate for fitness costs of certain mutations. The requirement for multiple mutations for high-level resistance likely explains the relatively slow emergence of AZT resistance in patients and during selection in cell culture.[145,238] Based on sequencing of virus isolated from patients following cessation of AZT therapy or in individuals newly infected with resistant virus, and of virus from replication competition experiments *in vitro*, AZT-resistant mutants appear to be modestly less fit than wild-type virus.[97]

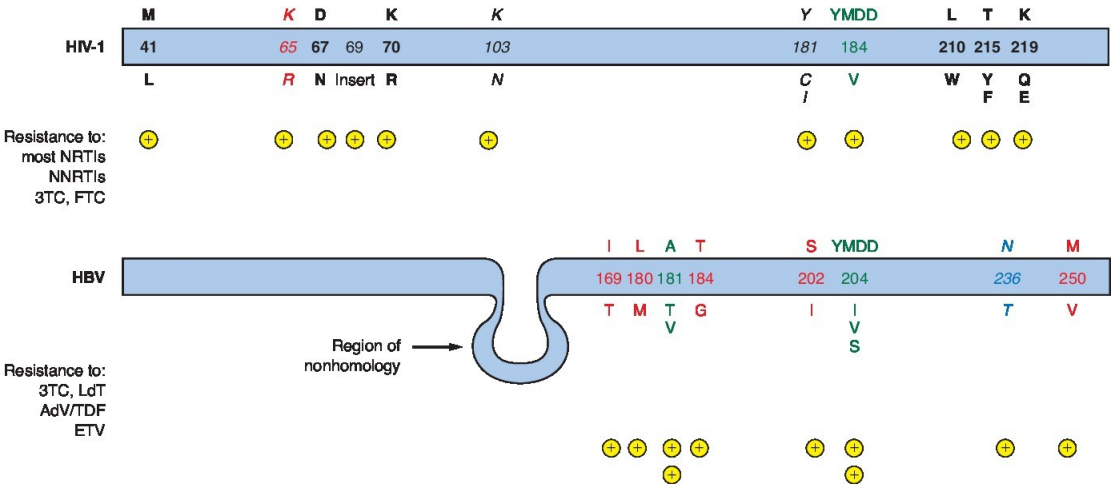

FIGURE 14.9 **Locations of drug resistance alterations in the HIV-1 and HBV polymerases**. The HIV-1 reverse transcriptase (RT) (*top*) and the HBV DNA polymerase (*bottom*) are related enzymes with sequence and predicted structural homology and are cartooned as bars. The HBV enzyme has a segment that is unrelated to the HIV enzyme, which is depicted here as looping out. The positions of selected substitutions and insertions that confer drug resistance are indicated in single letter code with the wild-type residue above the bar, the residue number within the bar, and mutant residues or inserts below the bar. Methionine 184 in HIV RT and methionine 204 in HBV polymerase are embedded within a YMDD motif (*green*) that is important for polymerase catalysis. Alterations in this methionine confer resistance to 3TC and related L-stereoisomer drugs, as does alteration of alanine 181 in HBV polymerase (also *green*). Boldfaced residues in HIV RT indicate the positions of mutations that affect susceptibility to most anti-HIV nucleoside analogs. An insertion at residue 69 in HIV RT (*blue*) together with other alterations including M41L, K70R, L210W, T215Y, and K219Q also affect susceptibility to these drugs. Substitution of arginine for lysine 65 (*purple*) in HIV RT confers resistance to all nucleoside analogs except AZT. Substitutions of lysine 103 and tyrosine 181 (*italics*) confer resistance to nonnucleoside reverse transcriptase inhibitors. Alterations of isoleucine 169, threonine 184, serine 202, or methionine 250 (*orange*) together with a leucine to methionine substitution at residue 180 (*orange*) plus a methionine 204 substitution in HBV polymerase can result in entecavir resistance. A threonine for asparagine 236 substitution in HBV polymerase (*blue*) can result in resistance to adefovir (high-level) and tenofovir (intermediate).

The mechanism of AZT resistance stems from chain elongation by 3′-5′ phosphodiester bond formation being a reversible process, so that in the presence of pyrophosphate or ATP, RT can catalytically excise the terminal nucleotide by a process termed pyrophosphorolysis (Fig. 14.10).[12,302] Given the K_m's of pyrophosphate and ATP for this reaction, and the concentrations of these species in cells, ATP is the likely substrate *in vivo*. The viral DNA chain is now free to resume elongation with the removal of the chain terminating nucleoside analog. AZT resistance mutations facilitate this reverse reaction of excision repair.[252,303] Crystal structures of wild-type and AZT-resistant RTs bound to either AZT-TP or the excision product indicate that resistance mutations alter the way that the AMP moiety of the excision product binds without affecting the AZT-TP–binding site.[443]

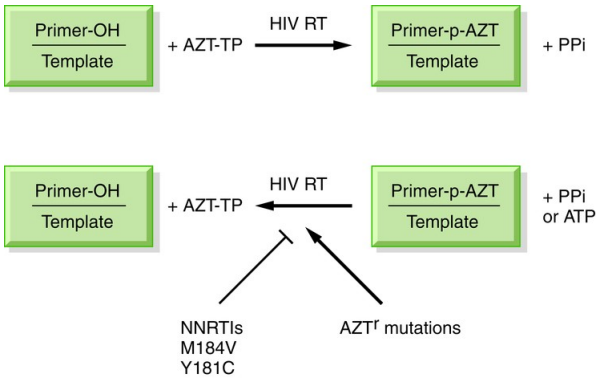

FIGURE 14.10 **Biochemical mechanism of resistance to AZT**. During synthesis of HIV DNA by HIV RT, AZT-TP is incorporated into the growing primer strand (primer-p-AZT), generating pyrophosphate (PPi). This reaction is reversible, so that RT can excise AZT-MP from the primer terminus and combine it with PPi, regenerating AZT-TP and primer/template. ATP can substitute for PPi in this excision reaction by donating two phosphates to AZT-MP. The excision reaction is enhanced by AZT resistance mutations but impaired by NNRTIs and certain HIV RT substitutions that confer resistance to 3TC (eg, M184V) and NNRTIs (eg, Y181C).

This resistance mechanism of excision repair applies to most, if not all anti-HIV nucleoside analogs; thus, these mutations have been termed *nucleoside-associated mutations* (NAMs) and account for many examples of cross resistance of HIV toward various nucleoside analogs.[462] Regardless of mechanism, various combinations of mutations affecting HIV RT can confer resistance to every approved nucleoside analog (Fig. 14.9; Ref.[207]).

The limited clinical effectiveness of AZT and problems with toxicity and resistance led to the development of other anti-HIV drugs and the use of combination chemotherapy.

Lamivudine and Emtricitabine

Lamivudine (3TC) was reported to exhibit anti-HIV activity in 1992.[78,79,391] Lamivudine and its close relative emtricitabine (FTC) appear to exhibit the least toxicity of the anti-HIV nucleoside analogs. This may be related to their unusual sugar moiety that contains a sulfur atom and is an l-stereoisomer, not the standard d-stereoisomer of normal nucleosides (Fig. 14.7C). Lamivudine is sequentially phosphorylated by cellular enzymes to its triphosphate.[54] Like AZT-triphosphate, 3TC-triphosphate is both a competitive inhibitor and a substrate for RT, and once incorporated, is an obligate chain-terminator of DNA synthesis. It is a more potent inhibitor of HIV RT than cellular polymerases.[171] The relative lack of toxicity of 3TC may be due to the negligible inhibition of host polymerases including mitochondrial DNA polymerase by 3TC-triphosphate.[171]

High-level resistance to 3TC develops rapidly both in cell culture and in patients treated with this drug alone (reviewed in Ref.[100]). A single mutation at codon 184 from Met to Val (M184V) or to Ile (M184I) confers a high degree of resistance to 3TC. M184 is within the conserved YMDD motif, in which the two Asp (D) residues are involved in catalysis of polymerization (Fig. 14.9). Both mutants are less fit than wild-type virus, which may be due to decreased processivity, primer usage, or initiation by the mutant enzyme or some combination of these (reviewed in Ref.[100]). The crystal structure of a 3TC-resistant RT was used to develop a molecular model that posits that codon 184 mutations result in steric hindrance that obstruct incorporation of 3TC-triphosphate, but not normal nucleotides.[386]

The M184V mutation has a number of other interesting effects, including conferring low-level resistance to some (eg, abacavir, Fig. 14.6) or hypersensitivity to other nucleoside analogs (eg, AZT and tenofovir; reviewed in Ref.[100]). This hypersensitivity to AZT and other drugs in the absence of other mutations is also observed in the presence of known drug resistance mutations such that levels of resistance are reduced with the addition of M184V. This phenotypic suppression is best explained by the M184V mutation reducing the excision repair of AZT-terminated primers (Fig. 14.10; Ref.[100]). Nevertheless, an accumulation of multiple mutations can result in high-level resistance to both AZT and 3TC, and other single mutations such as K65R can confer resistance to 3TC (and most other nucleoside analogs) (Fig. 14.9; Ref.[304]).

The mechanisms of action and resistance of FTC are very similar to those of 3TC; however, it may be slightly more potent and have a longer intracellular half-life.[369] Its coformulation with tenofovir (see below) has made this combination a backbone of combination antiretroviral therapy.

Other Anti-HIV Nucleoside Analogs

There have been numerous other anti-HIV nucleoside analogs approved over the years, but several of them have been discontinued. Two that are still used are abacavir and tenofovir. Abacavir contains an altered guanosine and an altered sugar that lacks both 2′ and 3′ OH and the ether oxygen (Fig. 14.7C). Following uptake into cells, abacavir is phosphorylated to its monophosphate, and then the 6-cyclopropylamino alteration on the base is removed, resulting in guanine linked to the modified sugar (carbovir), followed by further phosphorylation to the triphosphate.[124] Abacavir is also notable as an example of the use of pharmacogenomic tests to exclude patients likely to suffer adverse events, in this case those with the HLA-B*-5701 allele, which predisposes to a severe hypersensitivity reaction.[276] Tenofovir is an acyclic deoxyadenosine monophosphate analog (ie, a nucleotide analog) lacking a 3′OH and the entire 2′ moiety (Fig. 14.7C). It is administered as an orally available prodrug, either tenofovir disoproxil or tenofovir alafenamide (structures of these prodrugs not shown). These formulations have the advantage of being administered just once daily, making them attractive as constituents of long-term combination therapies.

Anti-HBV Nucleoside Analogs

Five nucleoside analogs—lamivudine (3TC), tenofovir, telbivudine, adefovir, and entecavir—have been approved for the treatment of HBV. Lamivudine and tenofovir have been described above under anti-HIV nucleoside analogs. Telbivudine is simply l-thymidine, thus sharing this isomeric configuration with 3TC and FTC, but has been withdrawn so will not be discussed further. Adefovir, like tenofovir, is a nucleotide analog with an adenine linked to an altered sugar similar to that in acyclovir, but containing an additional methylene moiety (Fig. 14.7C). Also, like tenofovir, it is administered as an orally available prodrug, in this case as adefovir dipivoxil. Entecavir is an unusual deoxyguanosine nucleoside analog in which the ether oxygen of the sugar is replaced with an exo carbon-carbon double bond (Fig. 14.7C). The mechanisms of action of anti-HBV nucleoside analogs are very similar to those of anti-HIV nucleoside analogs: They are converted to triphosphates by cellular enzymes, are incorporated into the growing DNA chain by HBV DNA polymerase (also a reverse transcriptase), and cause chain termination. Notably entecavir is a nonobligate chain terminator. Termination occurs two or three bases after its incorporation,[392] but precisely how this occurs remains unclear. Of note, entecavir-triphosphate has a higher affinity for HBV polymerase than does dGTP.[392]

The similarities in mechanisms of action of these drugs against the two viruses extend to some similarities in mechanisms of resistance but also important differences.[270] For example, a mutation in the methionine codon in the HBV *pol* gene that corresponds to codon M184 in the HIV *pol* gene can confer high-level resistance to 3TC (Fig. 14.9); however, in this case, the methionine to isoleucine mutant retains fitness, whereas methionine to valine or serine substitutions are usually only found in the presence of additional mutations. There is little if any evidence for HBV drug resistance mutations resulting in increased excision of incorporated drug as do AZT resistance mutations of HIV; rather, the mutations appear to affect incorporation of drug-triphosphate by various mechanisms. Interestingly, drug resistance that requires only a single mutation develops relatively quickly, but still more slowly *in vivo* with HBV than with HIV. In at least some cases, this may be because sites of mutation also encode residues in an overlapping reading frame that affect HBV surface antigen.

As is the case with AZT resistance, multiple mutations are required to confer high-level resistance to entecavir, and some of these mutations confer little resistance on their own.[270] This correlates with slow development of resistance *in vivo*. The greater potency and slower rates of resistance to entecavir and tenofovir has led to these drugs becoming preferred for treatment of HBV.[270] FTC and tenofovir are often used in patients who are infected with both HIV and HBV, because they are active against both viruses (even though FTC is not FDA approved for treating HBV infections).

Anti-RNA Virus Nucleoside Analogs

With all the nucleoside analog drugs against DNA viruses and retroviruses, it may seem surprising that there are so few anti-RNA virus nucleoside analogs. One possible contributor to this outcome may be the higher concentrations of competing ribonucleotides in cells relative to deoxyribonucleotides. Nevertheless, there is one long-standing nucleoside analog, ribavirin, that was approved for use against certain RNA

viruses; sofosbuvir, which is a mainstay anti-HCV drug; and now, two new ones, molnupiravir (see above) and remdesivir, which have received emergency authorization against SARS-CoV-2.

Ribavirin

Ribavirin is a nucleoside analog unlike the other nucleoside analogs discussed thus far in that it has a normal D-stereoisomer sugar (ribose) attached to a baselike moiety (Fig. 14.7D). It exhibits activity against many viruses in cell culture, but it has been approved only as monotherapy for severe respiratory syncytial virus (RSV) infections, and in combination with interferons for chronic HCV infections. Its routine use against RSV is not recommended except in certain complicated cases,[444] and its use against HCV has been superseded by newer, much more effective drugs. In both cases, it is not clear if it exhibits meaningful antiviral activity in patients.[41,99] Moreover, even in cell culture, its antiviral mechanism of action against RSV and HCV is still not well understood with multiple mechanisms proposed, including "error catastrophe,"[106] which, as discussed above, is a leading hypothesis for the mechanism of molnupiravir. However, in HCV replicons, ribavirin resistance was found to be due to mutations affecting a protein other than polymerase,[348] and studies of viral mutagenesis in patients treated with ribavirin have not provided definitive answers.[277,345] Further investigations of the mechanisms of ribavirin action and resistance may yield interesting new insights into virus biology and biochemistry.

Sofosbuvir, an Anti-HCV Nucleoside Analog

Sofosbuvir is a prodrug of β-D-2′-deoxy-2′-R-fluoro-2′-β-C-methyluridine monophosphate. Its corresponding nucleoside was initially identified as a metabolite of another drug[313,407] but did not show appreciable activity in cells, potentially due to slow formation of the nucleoside monophosphate.[275,407] Compound optimization efforts, therefore, focused on cell permeable prodrugs of the nucleoside monophosphate that could be selectively activated in the liver to mitigate potential off target toxicity.[141,407] These efforts lead to the discovery of the phenoxy phosphoramidate prodrug sofosbuvir[407] (Fig. 14.7D).

Postcellular uptake, the prodrug is cleaved by either carboxyesterase 1, or cathepsin A, and subsequently undergoes an intermolecular attack to release phenol.[314] Generation of the active nucleoside triphosphate is completed through hydrolysis of the alaninyl moiety to generate the monophosphate by histidine triad nucleotide-binding protein1 (Hint1), followed by di- and triphosphorylation by uridine/cytidine monophosphate kinase nucleotide diphosphate kinase, respectively.[141,314] Biochemical studies demonstrated sofosbuvir triphosphate is a selective substrate/inhibitor of the HCV RNA–dependent RNA polymerase vs human DNA and RNA polymerases.[11,235,275,313] Following incorporation into the growing RNA strand, sofosbuvir acts as a nonobligate chain terminator, terminating extension without addition of other nucleotides,[275] but how it induces termination is unclear. In cellular systems, sofosbuvir demonstrates similar activity across genotypes with 20-40 nM EC_{50} values in genotype 1-3 replicon assays.[234,235,374] *In vitro* resistance selection with sofosbuvir and nucleosides with a similar structure identified S282T mutation within the polymerase, which causes a 10-fold decrease in replicon sensitivity to sofosbuvir in the genotype 1 background.[234] A more complex resistance picture emerged upon resistance selection in the genotype 2 background where T179A, M289L, I293L, M434T, and H479P mutation were observed in addition to S282T; these mutations in combination with S282T were required to generate significant resistance to sofosbuvir.[234] Decreased replication fitness was observed with the S282T mutation across genotypes.[234] Treatment emergent clinical resistance to sofosbuvir is extremely rare consistent with a high barrier to resistance.[244,426] Sofosbuvir is a component of several HCV therapies (see under *Therapy of Viral Hepatitis*).

Remdesivir, an Anti–SARS-CoV-2 Nucleoside Analog

Remdesivir is a prodrug of 1′cyano 4-aza-7,9-dideazaadenosine (Fig. 14.7D). The compound was initially identified by examining a series of modified 1′-substituted 4-aza-7,9-dideazaadenosine C-nucleosides.[69] To improve cell permeability and potency, the nucleoside was converted to the monophosphoramidate prodrug.[69,457] Subsequently, remdesivir demonstrated <100 nM potency against SARS-CoV-2 in cell-based assays[75,455,488] and demonstrated efficacy in a nonhuman primate model of SARS-CoV-2 infection.[470]

Postcellular uptake, remdesivir is converted to its triphosphate (RTP) through a multistep mechanism. Initially, esterase cleavage leads to release of a free carboxylate, which cyclizes liberating the phenoxide. The resulting cyclic anhydride hydrolyzes to an alanine intermediate that is subsequently converted to the nucleoside monophosphate via phosphoramidase-mediated cleavage and then phosphorylated by cellular nucleotide kinases to yield RTP.[112,195,402] Mechanistic studies show that RTP is an excellent substrate and is incorporated selectively and rapidly by viral RdRps as an ATP analog, with kinetics similar and perhaps superior to those of ATP.[93,156] The efficiency of incorporation likely allows remdesivir to retain antiviral activity despite the presence of the coronavirus proof-reading exonuclease.[4] After incorporation of remdesivir, in ensemble assays, RNA strand elongation proceeds for 3-4 additional nucleotides before stalling; thus, remdesivir has been described as a delayed nonobligate chain terminator, although some elongation can be observed past the initial stall point, especially in the presence of higher concentrations of NTPs.[156,222] Interestingly, in single molecule studies, where numerous individual RdRps can be assessed, elongation following incorporation of remdesivir was found to proceed for as many as 30 additional nucleotides, followed by pausing and then diffusion backward of the RdRp, which led to the proposal that remdesivir incorporation leads to dissociation of the product strand in a process termed backtracking,[393] which may be promoted by the SARS-CoV-2 exonuclease.[222] For either proposed mechanism, cryoEM, mutational, and biochemical studies suggest that stalling arises from a translocation barrier where the C-1′nitrile of remdesivir sterically clashes with serine 861 of the RdRp when remdesivir is incorporated into the appropriate location (N-4) in the primer strand, precluding addition of the subsequent nucleotide and rendering the 3′ end of the primer less susceptible to the SARS-CoV-2 exonuclease.[156,222,435,456] It has also been suggested that remdesivir acts by incorporation into template, where it causes reduced UTP incorporation opposite its template position.[435]

In vitro resistance selection conducted against SARS-CoV-2 have resulted in substitutions in the viral polymerase that increase the EC_{50} in cells by roughly 2- to 10-fold.[413,429] One mutation is hypothesized to cause a structural rearrangement, which decreases the steric clash preventing binding of the remdesivir at the N-4 site postincorporation.[429] Others affect binding or incorporation of RTP.[413] The mutations are associated with a decrease in viral fitness and are not observed in sequence data bases of isolates.[413,429] Clinical resistance to remdesivir in treatment of COVID-19 has been rare, though a case study of an immunosuppressed patient whose infection rebounded during and after remdesivir treatment noted the emergence of virus with ~sixfold resistance and reduced fitness.[143]

Nonnucleoside Inhibitors of Polymerases

As described above, viral polymerases are excellent targets for antiviral drugs, and nucleoside analogs, for all their successes, can inhibit cellular as well as viral enzymes, and viruses can mutate to resist these drugs. As a result, efforts have been made to discover compounds that inhibit viral polymerases by other mechanisms.

Foscarnet, an Antiherpesvirus Nonnucleoside Polymerase Inhibitor

The first of these to be approved for clinical use was foscarnet (phosphonoformic acid, PFA; structure in Fig. 14.11). Although it is not orally available so that intravenous administration is required, and it is nephrotoxic, it is approved for treatment of severe HSV, VZV, and HCMV infections that are resistant to front-line drugs.

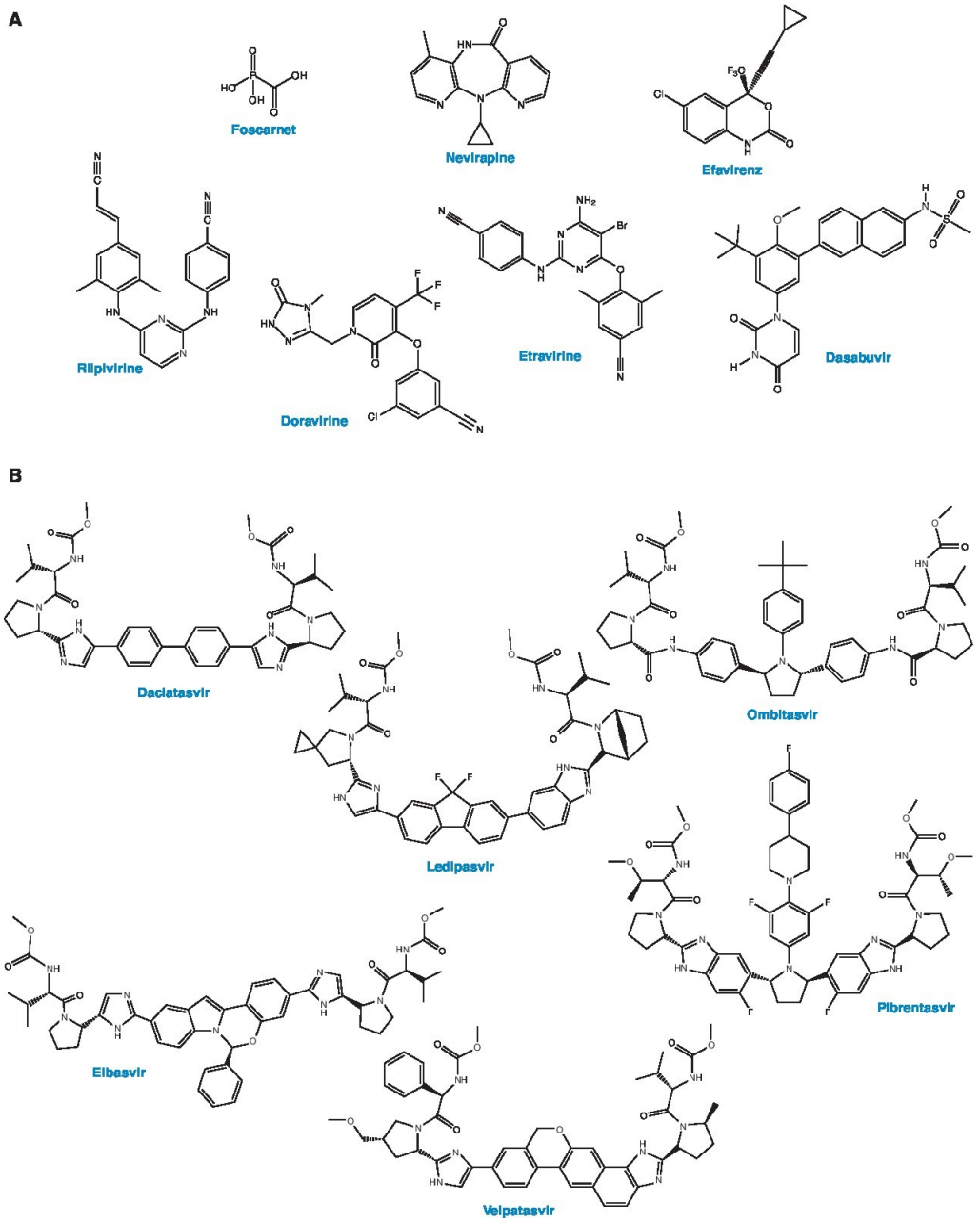

FIGURE 14.11 **Nonnucleoside DNA polymerase inhibitors (foscarnet and NNRTIs) and NS5A inhibitors**. **A**. Foscarnet is a pyrophosphate analog that inhibits viral polymerases. It is approved for treatment of HSV, VZV, and HCMV infections that are resistant to other drugs. NNRTIs, nevirapine, efavirenz, etravirine, rilpivirine, dasabuvir, and doravirine, which do not mimic any known ligands of DNA polymerases, inhibit HIV-1 reverse transcriptase and are approved for treatment of HIV-1 in combination with other drugs. **B**. Daclatasvir, ledipasvir, ombitasvir, elbasvir, velpatasvir, and pibrentasvir bind to and inhibit HCV NS5A and are approved for treatment of HCV in combination with other drugs. (Modified with permission from Li JZ, Coen DM. Pharmacology of viral infections. In: Golan DE, Armstrong EJ, Armstrong AW, eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. 4th ed. Wolters Kluwer; 2017:694-722.)

Foscarnet is an analog of pyrophosphate, which is a product of polymerization of nucleic acids. Unlike the nucleoside analogs described above, it does not require activation by either cellular or viral enzymes but rather inhibits herpesvirus DNA polymerases directly and selectively.

Inhibition is *not* competitive with deoxynucleoside triphosphates. Rather, it appears that foscarnet acts as a product analog, preventing normal pyrophosphate release so the polymerase cannot complete the catalytic cycle.[119,332] The structure of foscarnet bound to a DNA polymerase that is a chimera of a bacteriophage DNA polymerase and a segment of HCMV polymerase that is important for foscarnet sensitivity has been determined.[487] In the structure, the drug binds to highly conserved basic residues in a closed form of the enzyme that has not translocated to the next base on the template. The drug occupies the position of the beta and gamma phosphates of an incoming dNTP. The structure suggests that foscarnet may stabilize this closed, untranslocated state, thus stalling the polymerase.

Because foscarnet is not a nucleoside analog, HSV and VZV *tk* mutants and HCMV *UL97* mutants are not resistant to it. However, although foscarnet inhibits DNA polymerase by a mechanism that differs substantially from the nucleoside analogs, many *pol* mutants that are resistant to nucleoside analogs are resistant to foscarnet. Moreover, most foscarnet-resistant mutants are resistant to one or more nucleoside analogs (reviewed in Ref.[153]). This has been an impetus to further drug development.

Anti-HIV Nonnucleoside Reverse Transcriptase Inhibitors

There are currently six FDA-approved nonnucleoside reverse transcriptase inhibitors (NNRTIs)—efavirenz, nevirapine, etravirine, rilpivirine, dasabuvir, and doravirine (Fig. 14.11)—that are used for treatment of HIV infections. The first NNRTIs were discovered using high-throughput screening for inhibition of HIV-1 RT (eg, nevirapine[300]). The compounds that emerged were then chemically modified to improve their antiviral activities, stabilities, and pharmacokinetics and reduce their toxicities. The NNRTIs are highly specific, inhibiting HIV-1 RT at low concentrations while not inhibiting human DNA polymerases or even the RT of the closely related virus HIV-2.[300] This biochemical selectivity is reflected by the high-level resistance conferred by HIV-1 *pol* mutations.[368] These drugs directly inhibit RT. The NNRTIs do not mimic a natural ligand of a polymerase. Each NNRTI appears to share a polycyclic structure that tends to form a “butterfly wings” conformation upon binding to RT. NNRTIs bind to a pocket near the catalytic Asp residues of RT.[220] This pocket is not observed in structures of RT in the absence of drug but rather is stabilized by drug binding.[375] Thus, NNRTI binding causes a substantial conformational change in RT, including distortion of the catalytic Asp residues, and slows the enzyme's incorporation of dNTPs into DNA.[373,408] NNRTIs inhibit pyrophosphorolysis and the excision of AZT from primer termini by RT (Fig. 14.10; see Ref.[326]), which likely accounts for synergy of these drugs against HIV.[367]

As with 3TC, resistance to NNRTIs develops quickly both *in vitro* and in patients, with single amino acid substitutions conferring high-level resistance.[296,368,371] Some common resistance mutations arise in codon 181 (Fig. 14.9), changing Tyr to Cys (Y181C) or to Ile (Y181I), and cause high-level resistance.[296,368] This change alters a residue known to interact with NNRTIs[86,220,478] and thus reduces drug binding. The Y181C mutant is relatively fit in infected cells[87,200] and in patients. Some but not all other NNRTI-resistant mutants exhibit reduced fitness in cells and in patients.[264] The Y181C mutation, like the M184V mutation, sensitizes otherwise AZT-resistant mutants to AZT due to suppression of excision repair (Fig. 14.10, see Ref.[394]). The newest NNRTI, doravirine, retains activity against Y181C and certain other common NNRTI-resistant mutants.[127] The different mechanisms of action and resistance of the anti-HIV nucleoside analogs and NNRTIs help provide the rationales for various combinations of these drugs in treating HIV (see section on *HIV Therapy*).

Inhibitors of Hepatitis C Virus NS5A Protein

The evolution of NS5A inhibitors for the treatment of HCV followed a similar path to what was previously described for NS3/4A inhibitors. To date, six NS5A inhibitors have been approved by the U.S. FDA (Fig. 14.11B). The first generation of approved therapies (daclatasvir, ledipasvir, and ombitasvir) were components of some of the first all oral regimens for the treatment of genotype 1 HCV. The main liabilities of these compounds were resistance profile and the lack of pangenotypic coverage. These molecules were rapidly supplanted by molecules with pangenotypic activity and superior resistance profiles (elbasvir, velpatasvir, pibrentasvir).

NS5A inhibitors were originally discovered through a one million compound screen in cell culture for activity against genotype 1b genome replication using a subgenomic replicon assay, which discovered a series of molecules whose activity could not be related to inhibition of the known enzymatic targets such as polymerase or protease.[31,144] Selection and identification of resistance mutations suggested that the target was the viral protein NS5A.[144,251] NS5A is essential for viral replication but does not have a known enzymatic function.[310,344,381]

Despite extensive study, particularly on daclatasvir, the mechanism of action of NS5A inhibitors is not fully understood. To date, resistant mutants to NS5A inhibitors have all contained alterations within the first 100 amino acids of NS5A within domain one, which forms a symmetrical dimer capable of RNA binding, contains an amphipathic alpha helix that anchors the protein to cellular membranes, and plays a role in genome replication.[13,192,310,381,437] Biochemical studies demonstrated that daclatasvir can bind to domain one with nM affinity, and that binding is diminished by a resistance mutation (Y93H) or the addition of RNA.[13] Furthermore, NS5A inhibitors can disrupt the formation of subcellular structures of the “membranous web,” a subcellular structure associated with formation of the replication complex.[33] Synergy has been noted with different NS5A inhibitors, including the ability to rescue daclatasvir susceptibility in the presence of the Y93H mutation using a second NS5A inhibitor that shows no activity as a single agent.[419] These data along with studies demonstrating, remarkably, that NS5A is present in vast molar excess to NS5A inhibitors at their effective concentrations in infected cells have led to the suggestion that the inhibitors may act to disrupt the function of NS5A oligomers or distinct pools of NS5A in different complexes.[325,419]

Both the first- and second-generation NS5A inhibitors share a similar molecular topology, containing a hydrophobic core flanked by two largely identical substituents whose symmetry reflects the dimer structure of NS5A (Fig. 14.11B). The second common pharmacophoric feature is a chiral substituted pyrrolidine terminating in an isopropyl carbamate. In all cases, the molecules are larger and more hydrophobic than traditional oral small molecule therapies. NS5A inhibitors are among the most potent small molecule antivirals that have been developed to date with potencies in subgenomic genotype 1 replicon assays below 31 pM for all of the approved compounds.[65,80,144,228,267,318,474]

Resistance substitutions selected in cell culture are relatively consistent across genotypes and include M28T/V, Q30E/H/K/R, L31M/V, H58D, Y93C/H/N, and well as highly resistant Q30, Y93 double mutants. Mutations at positions 28, 31, and 93 are the most prominent. Complex interactions between mutations have been shown to drive high-level resistance even when a single mutation conferred little drug resistance on its own.[324] Sequencing of virus in patients treated with various NS5A inhibitors as a single agent, whose virus loads rebounded, showed a similar constellation of mutations with common mutational hot spots observed at position M28, Q30, L31, and Y93.[136,228,246,267,474] Notably,

the majority of genotype 2 HCV patients possess the L31M polymorphism at baseline,[492] which helped drive development of second-generation NS5A inhibitors. Optimization of the next-generation NS5A inhibitors, such as pibrentasvir and velpatasvir, led to molecules that exhibit sub nM to low pM EC_{50} values against wild-type replicons across all 6 genotypes, including genotype 2 replicons possessing the L31M polymorphism and dramatic improvements in the resistance profile including against Y93H.[318,324]

All of the NS5A inhibitors are used clinically in combination with another antivirals.

Inhibitors of HIV Integration

Inhibitors of HIV Integrase

A crucial stage in genome replication of HIV and other retroviruses is integration of the linear, double-stranded DNA product of reverse transcription into the host genome. The enzyme that carries out this step, integrase, is an essential enzyme. Integrase assembles onto sequences from the ends of HIV DNA, cleaves dinucleotides from each 3′ strand, and transfers these strands to covalently link them with target DNA (Fig. 14.12A). The catalytic core of integrase includes three conserved acidic residues called the DDE motif (Fig. 14.12C), which binds Mg^{++} that is essential for integrase activity. Discovery of integrase inhibitors was propelled by the development of an assay selective for DNA strand transfer,[180] which identified inhibitors that act much more potently at this step than at prior steps (Fig. 14.12A). Medicinal chemistry studies of various classes of diketoacid scaffolds led to the development of raltegravir (Fig. 14.12B).[418] Its diketoacid moiety binds to the metal coordinated by the DDE motif, and additional moieties bind to the enzyme and, evidently, DNA (Fig. 14.12C).[7,64,292] Raltegravir was tested against a variety of enzymes that are Mg^{++} dependent (eg, HIV RT) and was highly selective for integrase. Its selectivity for the strand-transfer step seems to depend on binding to a conformation of integrase that is stable only after processing of 3′ ends.

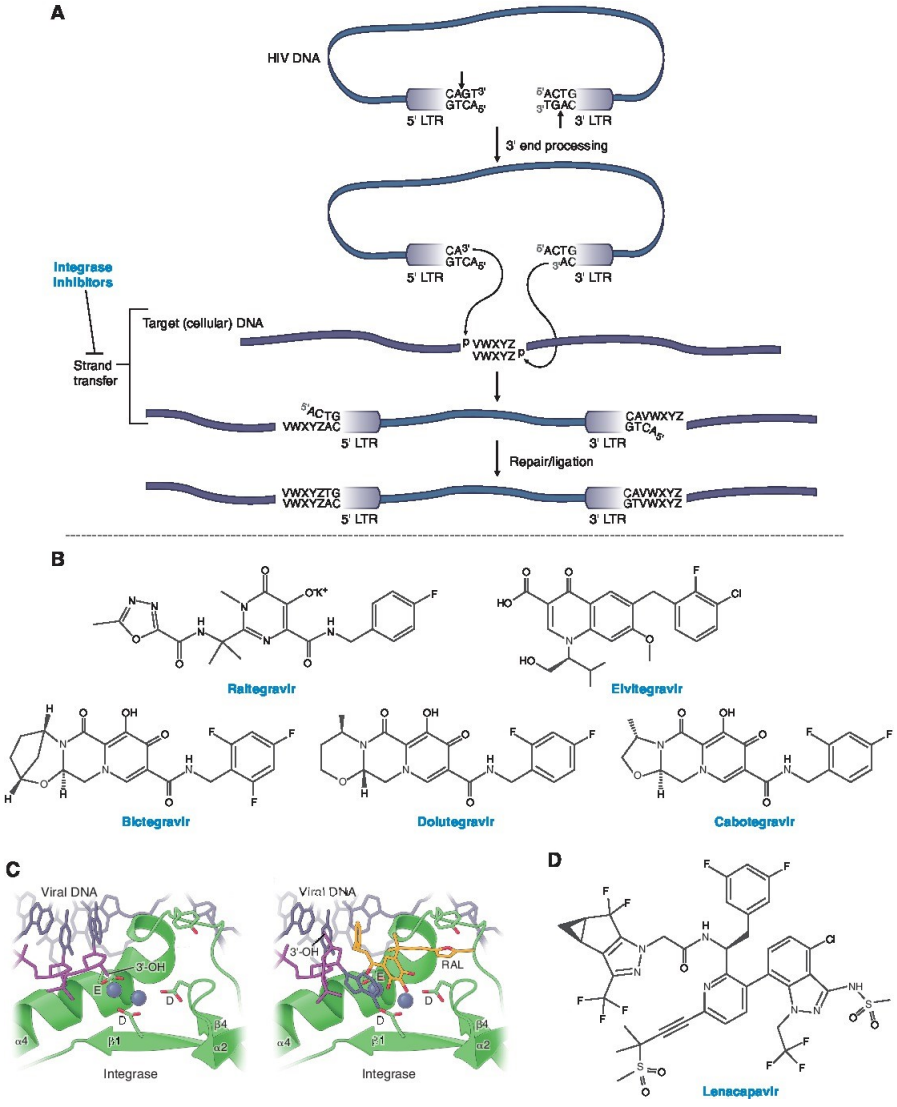

FIGURE 14.12 **HIV integrase, raltegravir action, and lenacapavir**. **A**. Linear, double-stranded HIV DNA, generated by reverse transcription, contains a four base sequence at each of its ends, as part of its long terminal repeat (LTR). Integrase (IN), which contains an N-terminal zinc-finger domain important for DNA binding, a C-terminal domain, and an internal core domain containing three acidic residues (DDE) that coordinate magnesium ions important for catalysis, binds to HIV DNA (as part of a larger complex), and cleaves a dinucleotide from the 3′ strand at each end of viral DNA (3′ end processing). The integrase-viral DNA complex then catalyzes the attack of the recessed 3′ ends of viral DNA on phosphodiester bonds of target (cellular) DNA (strand transfer). The product results in 5′ flaps of viral DNA and gaps in cellular DNA. Integrase releases and host enzymes remove the flaps and repair the gaps, resulting in integrated

viral DNA flanked by duplicated host sequences. Raltegravir inhibits the strand transfer step of this process. **B**. Structures of raltegravir, elvitegravir, bictegravir, dolutegravir, and cabotegravir. **C**. The mechanism by which raltegravir inhibits integrase. The left panel shows the active site of a retroviral integrase bound to viral DNA in the absence of drug, while the right panel shows the same active site in the presence of raltegravir. The integrase protein is shown in *green* with alpha helices shown as helical ribbons and beta-strands as arrows. Asp and Glu residues that coordinate magnesium ions, the viral DNA, and raltegravir (RAL) are shown as stick models, and the magnesium ions as *gray* spheres. The 3′ hydroxyl group of DNA is adjacent to the magnesium ions in the absence of drug but angled away from the ions when raltegravir is bound. (This figure panel was kindly provided by Peter Cherepanov.) **D**. Structure of the capsid binding drug lenacapavir, which blocks HIV replication at the step of integration. (Modified with permission from Li JZ, Coen DM. Pharmacology of viral infections. In: Golan DE, Armstrong EJ, Armstrong AW, eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. 4th ed. Wolters Kluwer; 2017:694-722.; Freed EO, Martin MA. HIVs and their replication. In: Knipe DM, Howley PM, Griffin DE, et al., eds. *Fields Virology*. 5th ed. Wolters Kluwer Health/Lippincott Williams & Wilkins; 2007. Ref.[135])

Raltegravir inhibits HIV replication at low nanomolar concentrations. As expected, HIV mutants resistant to raltegravir contain mutations in sequences encoding the integrase, with most mutations that confer resistance altering residues close to the inhibitor binding site.[292] Of mechanistic interest, one mutation alters a residue that, in the crystal structure, makes contact with a nucleotide corresponding to the 5′ terminus of viral DNA. There are also mutations that do not themselves confer resistance but increase the effects of other mutations. Similar mutations are selected by raltegravir in cell culture and in patients. Single mutations seem to be sufficient to confer clinically significant resistance, but most of these mutations decrease HIV fitness at least modestly.[292]

Since the development of raltegravir, four other integrase strand-transfer inhibitors have been FDA approved—elvitegravir (administered with a cytochrome P450 inhibitor, cobicistat, to boost serum drug levels), dolutegravir, bictegravir, and cabotegravir (which can be administered as a long-acting injection) (Fig. 14.12B). Some of these drugs are less prone to resistance than raltegravir. Integrase inhibitors are now key components of several anti-HIV combination therapies and prophylaxes.

A Capsid-Binding Drug That Blocks HIV Integration—Lenacapavir

A different way of blocking HIV integration has been revealed by studies of a new drug, lenacapavir (Fig. 14.12D). This drug was approved by the FDA in late 2022 as an every 6-month injectable in combination with other antiretrovirals for treatment of otherwise untreatable HIV infections. Lenacapavir was developed starting from a small molecule that accelerates self-assembly of HIV capsids from capsid protein p24 monomers.[261,484] Structural information from X-ray crystal structures of various related small molecules bound to cross-linked hexamers of p24 was used to optimize potency and metabolic stability, and a similar structure of the p24 hexamer-lenacapavir complex revealed that lenacapavir binds to and bridges interfaces between p24 monomers.[34,261] Accordingly, it binds much more tightly to p24 hexamers (subnanomolar K_d) than to monomers, and this very tight binding translates to highly potent antiviral activity in cells (subnanomolar ED_{50}) accompanied by cytotoxicity only at much higher concentrations.[34,261]

Based on its ability to accelerate HIV capsid assembly, one might anticipate that lenacapavir could inhibit capsid disassembly and/or other functions during the uncoating stage and subsequent steps culminating in the integration step of the genome replication stage.[481] Indeed, lenacapavir stabilizes capsids within HIV particles[34] and inhibits multiple steps of HIV infection. Early steps are inhibited with much higher potency—similar to that seen for viral replication—than later steps such as capsid assembly and maturation.[34,261] At concentrations that block viral replication, the drug has little effect on production of viral reverse transcripts (cDNA), so does not block uncoating but does block integration.[34,261] This appears to be due, at least in part, to impairing the import of complexes that contain DNA, p24, and integrase into the nucleus, as there are reductions in the amounts of integrase and viral DNA in that compartment with concomitant increases in the cytoplasm.[34,261] These effects can be explained by inhibition of dissociation of these complexes but likely effects on interactions of complexes containing DNA, p24, and integrase with host factors important for transit to the nucleus, nuclear import, and/or integration likely also contribute.[34,261,481]

Lenacapavir resistance mutations have been selected in cell culture and map, as expected, to the sequences that encode the binding site for the drug on p24.[261] Interestingly, most affect highly conserved residues and reduce replication in cell culture except one that confers relatively modest resistance and also arose in a patient during a clinical study.[261] Further study of the mechanisms of lenacapavir resistance and its clinical impact are needed.

Prospects for New Inhibitors and Targets of Viral Genome Replication

Given the successful development of numerous antiviral drugs targeting multiple steps in genome replication, it is not surprising that work continues on finding new drugs against established targets and against new targets that function at this stage. New nucleoside analog and nonnucleoside inhibitors of viral polymerases and other replication enzymes continue to be developed. One that has progressed to phase 3 clinical trials is pritelivir. This drug acts on the trimeric helicase-primase complex of HSV, which unwinds DNA ahead of the DNA polymerase on the leading strand and lays down primer for the polymerase to extend on the lagging strand.[219]

Inhibition of Viral Assembly/Egress

The processes by which viruses assemble infectious virions and egress from cells are closely intertwined and are attractive targets for drug discovery because they are unique to virus biology. As will be detailed below, certain steps in assembly are crucial for egress and certain steps in egress are crucial for assembly. Moreover, certain steps in assembly can occur after egress and certain steps in egress can occur prior to completion of assembly. Thus, for the purposes of this chapter, assembly and egress are considered together as a single stage of infection. FDA-approved drugs that target different steps during this stage are letermovir targeting DNA packaging into HCMV capsids, maribavir targeting nuclear egress of HCMV prior to completion of assembly of the virion, tecovirimat targeting assembly of a poxvirus glycoprotein that is crucial for egress, various HIV protease inhibitors targeting the final step of assembly—maturation into infectious virions—which can occur following egress, and various influenza neuraminidase inhibitors targeting release of virus particles from the extracellular surface of cells.

Letermovir—an Inhibitor of HCMV DNA Cleavage During Packaging

A major step in the assembly of all viruses is incorporation of viral genomes into capsids. For herpesviruses, the current model (reviewed in Ref.[182]) posits that replicated viral DNA is in the form of long concatemers made up of unit-length monomers arranged head-to-tail, and that a closed precursor called the procapsid is formed from a set of capsid and scaffold proteins and a viral protease. Then, additional capsid proteins involved in DNA packaging are assembled onto the surface of the procapsid, viral DNA is inserted into the capsid through a portal at a capsid vertex in a process preceded or accompanied by protease cleavage of the scaffold protein, and then the concatemerized DNA is cleaved at the genome terminus to yield the mature nucleocapsid (also known as C-capsid) containing a unit-length linear DNA. The cleavage activity, known as terminase, has three subunits, all of which are essential for viral replication.

Several different drug classes have been shown to inhibit HCMV DNA packaging. One class is comprised of 3,4-dihydro-quinazoline-4yl-acetic acid derivatives that were identified in a high throughput screen for anti-HCMV activity. One of these derivatives, now known as letermovir (Fig. 14.13A), was found to have ~50 nM potency against HCMV replication with orders of magnitude higher concentrations required to observe cytotoxicity or antiviral activity against other viruses, including HSV.[262,284] The target was revealed by isolation of drug-resistant mutants, whose mutations mapped to the *UL56* gene, which encodes a subunit of the terminase[154] thought to interact with DNA sequences required for cleavage and packaging.[42,182] Consistent with inhibition of terminase, letermovir-treated infected cells contained wild-type levels of replicated DNA, but a large decrease in unit-length genomes.[154] Which specific step in cleavage/packaging is inhibited by letermovir is unclear, as it has been difficult to reconstitute these activities *in vitro*.

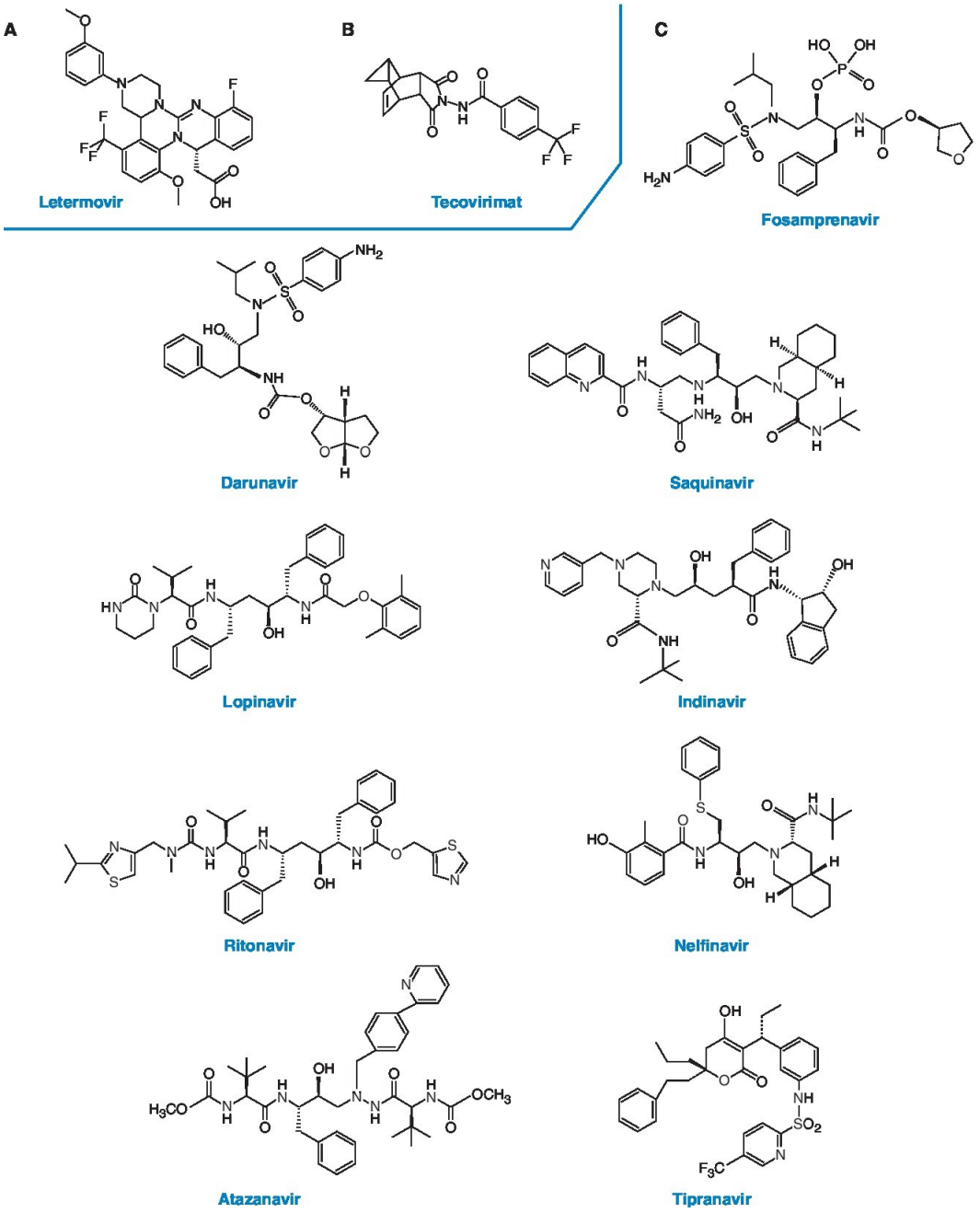

FIGURE 14.13 **Letermovir, tecovirimat, and anti-HIV protease inhibitors**. **A**. Structure of the anti-HCMV packaging inhibitor, letermovir. **B**. Structure of the antipoxvirus envelopment and egress inhibitor, tecovirimat. **C**. The structures of approved and marketed anti-HIV protease inhibitors fosamprenavir, darunavir, saquinavir, lopinavir, indinavir, ritonavir, nelfinavir, atazanavir, and tipranavir are shown.

Letermovir is orally available, causes less severe adverse events than older anti-HCMV drugs, and is active against most mutants resistant to such drugs.[262,287] HCMV mutants resistant to letermovir can evolve rapidly in cell culture with little effects on fitness.[70,72] Most such mutants have *UL56* mutations, but mutations in the *UL89* and *UL51* genes that encode different terminase subunits can confer slight resistance on their own and more substantially augment resistance conferred by *UL56* mutations.[71,72] Prophylaxis failures occurred in ~40% of treated patients,[287] but a relatively small fraction of those failures were associated with letermovir-resistant viruses, most of which had mutations in *UL56*.[104] At the time of this writing, work is ongoing to better understand the clinical importance of letermovir resistance, both in a prophylactic setting and in settings where the drug might be used to treat active HCMV infections, where resistance would be more likely to arise.

Maribavir, a Protein Kinase Inhibitor That Blocks HCMV Nuclear Egress

As summarized above in the section on inhibitors of genome replication, maribavir (Fig. 14.7B) is a nucleoside analog inhibitor of the protein kinase encoded by HCMV, UL97. Aside from inhibiting genome replication in nondividing cells, in both dividing and nondividing cells, maribavir (or a mutation inactivating UL97) also inhibits an unusual process during the assembly and egress of HCMV (and other herpesviruses), known as nuclear egress.[229,364] Indeed, in dividing cells, maribavir's inhibition of nuclear egress (~30-fold) is similar to its inhibition of virus yield.[229]

During herpesvirus nuclear egress, nucleocapsids migrate from their sites of assembly in the nuclear interior toward the nuclear periphery, where they cross the nuclear lamina that underlies the inner nuclear membrane, bud through the inner nuclear membrane, acquiring an envelope, and then fuse with the outer nuclear membrane to be released, envelope-less, into the cytoplasm.[36,273] During subsequent steps in assembly and movement through the cytoplasm, the capsid acquires a new envelope and envelope proteins, as well as tegument proteins that reside between the capsid and envelope. The completed virion can then egress from the cell.

What step in nuclear egress is inhibited by maribavir? During wild-type HCMV infection, the nuclear lamina becomes disrupted with gaps visible by confocal light microscopy, but in the presence of maribavir, the lamina remains intact.[168] Expression of active UL97 in cells following transfection also disrupts nuclear lamina,[283] and UL97 can phosphorylate the lamina component, lamin A/C *in vitro* on the site phosphorylated by cyclin-dependent kinase 1 that is crucial for disruption of nuclear lamina during mitosis.[168] Maribavir blocks phosphorylation at this site both *in vitro* and in HCMV-infected cells.[168] Expression of a dominant negative lamin A/C mutant that disrupts nuclear lamina in the place of UL97 alleviates the loss of UL97 function for replication in dividing cells.[396] Thus, the model is that maribavir blocks nuclear egress by inhibiting UL97 phosphorylation of lamin A/C, thereby preventing nucleocapsids from crossing the nuclear lamina. It remains unclear whether UL97 has functions other than disruption of the nuclear lamina during nuclear egress. Maribavir resistance is discussed in the section on this drug under *Inhibition of Viral Genome Replication*.

Tecovirimat—an Inhibitor of Envelopment and Egress

Concerns about the use of variola virus as a biological weapon led to efforts to develop antipox virus drugs. The first of these to achieve FDA approval (in 2018) was tecovirimat for treatment of smallpox disease. Tecovirimat (Fig. 14.13B) was discovered following a high throughput screen for inhibition of cytopathic effect induced by vaccinia virus. The screen identified a series of tricyclononine carboxyamides whose subsequent optimization resulted in tecovirimat.[22] Tecovirimat is potent against a variety of poxviruses including variola and vaccinia viruses in cell culture with EC_{50}s < 100 nM, and selective as it fails to inhibit other viruses or cell viability at concentrations more than 400-fold higher.[110,483] It also shows antiviral activity, and good tolerability and pharmacokinetics in animal models,[483] which form the basis for FDA approval as clinical trials for antivariola drugs are neither ethical nor practical.

Poxviruses assemble two kinds of infectious particles—intracellular virions, which have a single envelope, and are only infectious following mechanical lysis of cells, and, following wrapping of these particles with a second membrane, virions that can transit to the plasma membrane where the outer membrane fuses with the cell plasma membrane to release infectious virus that can infect neighboring cells and disseminate.[311] Interestingly, tecovirimat inhibits formation of extracellular infectious virus with little effect on intracellular virus.[483] The target of tecovirimat was revealed by the mapping of tecovirimat resistance mutations to the F13L gene of vaccinia virus, which encodes the major viral protein exclusively found in the outer membrane.[483] Inactivation of this gene results in a phenotype similar to that of tecovirimat—greatly reduced production of extracellular virions.[39] Interestingly, there is also evidence that those virions that are produced are less infectious,[49] but whether tecovirimat also has that effect is not yet known.

How tecovirimat inhibits the F13L gene product is unclear. The protein contains a motif that is conserved among a family of phospholipases, and substitutions in this motif blocks formation of the outer wrapping membrane and plaque formation.[378,420] Additionally, the protein has been reported to have lipase activity.[20] Tecovirimat treatment alters the localization of this protein as well as a second outer envelope protein.[110] Docking of tecovirimat to a putative model of the protein based on the structure of a bacterial phospholipase identified a potential pocket to which the drug binds; this predicted pocket also contains residues altered by tecovirimat resistance mutations.[110] It has been proposed that binding of drug to this pocket could affect a nearby loop-bearing residues in the motif conserved among phospholipases.[110] Further investigation is required to test this proposal and the putative model of the structure of the protein.

As noted, tecovirimat resistance mutations can arise in cell culture. The initial isolation of such mutants was obtained simply by plating virus in the presence of high concentrations of tecovirimat,[483] while a subsequent study found that multiple passages in the presence of drug were required.[110] Regardless, resistant vaccinia viruses that replicate as well as wild-type virus in cell culture were obtained although these mutants exhibited smaller plaque sizes. It would be interesting to know if these mutants had altered pathogenicity in animal models, which could shed light on possible outcomes of resistance in humans.

HIV Protease Inhibitors That Block Maturation

For many viruses, including HIV, mere assembly of proteins and nucleic acid into particles is not sufficient to produce an infectious virion. For such viruses, an additional step, maturation, is required. In most cases, these viruses encode proteases that are essential for maturation. The approved antiviral drugs that target HIV protease and are still marketed are saquinavir, ritonavir (which is now used mainly at low dose to boost

plasma drug levels of other protease inhibitors), lopinavir, fosamprenavir, indinavir, nelfinavir, atazanavir, darunavir, and tipranavir (Fig. 14.13C). This class of drugs is a paradigm of rational drug design.

The cleavage sites in the viral polypeptide substrates of HIV protease are conserved and somewhat unusual[347,354] and, unlike the human proteases most closely related to it, HIV protease is a symmetric homodimer, suggesting both specificity and a starting point for drug design.[316] For simplicity, only the development of ritonavir will be detailed. Ritonavir's design began with the recognition that a natural substrate of the protease contains a phenylalanine-proline (Phe-Pro) bond; mammalian enzymes rarely if ever cleave at such a site. The transition state of this substrate during cleavage was then modeled (Fig. 14.14A). Knowing that HIV protease is a symmetric dimer, an analog of the transition state was modeled, using the same residue, phenylalanine, on both sides, and with a CHOH group that mimics the transition state as the center of symmetry. This molecule, A-74702, was a very weak inhibitor of HIV protease, but adding groups symmetrically to both ends to form A-74704, resulted in a >40 000-fold increase in potency (K_i ~ 5 nM[118]). Attempts to modify A-74074 to improve solubility reduced potency, so a related potent inhibitor, A-75925, in which the center of symmetry was a C-C bond between two CHOH groups, became the scaffold for further additions to both ends.[215] This resulted in a soluble, highly potent inhibitor, A-77003 (Fig. 14.14B). However, this compound was not orally available.[214] It was modified by removing a central OH group and altering other moieties at each end (Fig. 14.14B). This resulted in less solubility but improved antiviral activity and good oral bioavailability.[213] These improvements took advantage of the X-ray structure of HIV protease complexed with each version of the inhibitors (reviewed in Refs.[10,472]). By examining these structures, chemists were able to make informed guesses about what groups to add or subtract. The result was ritonavir.

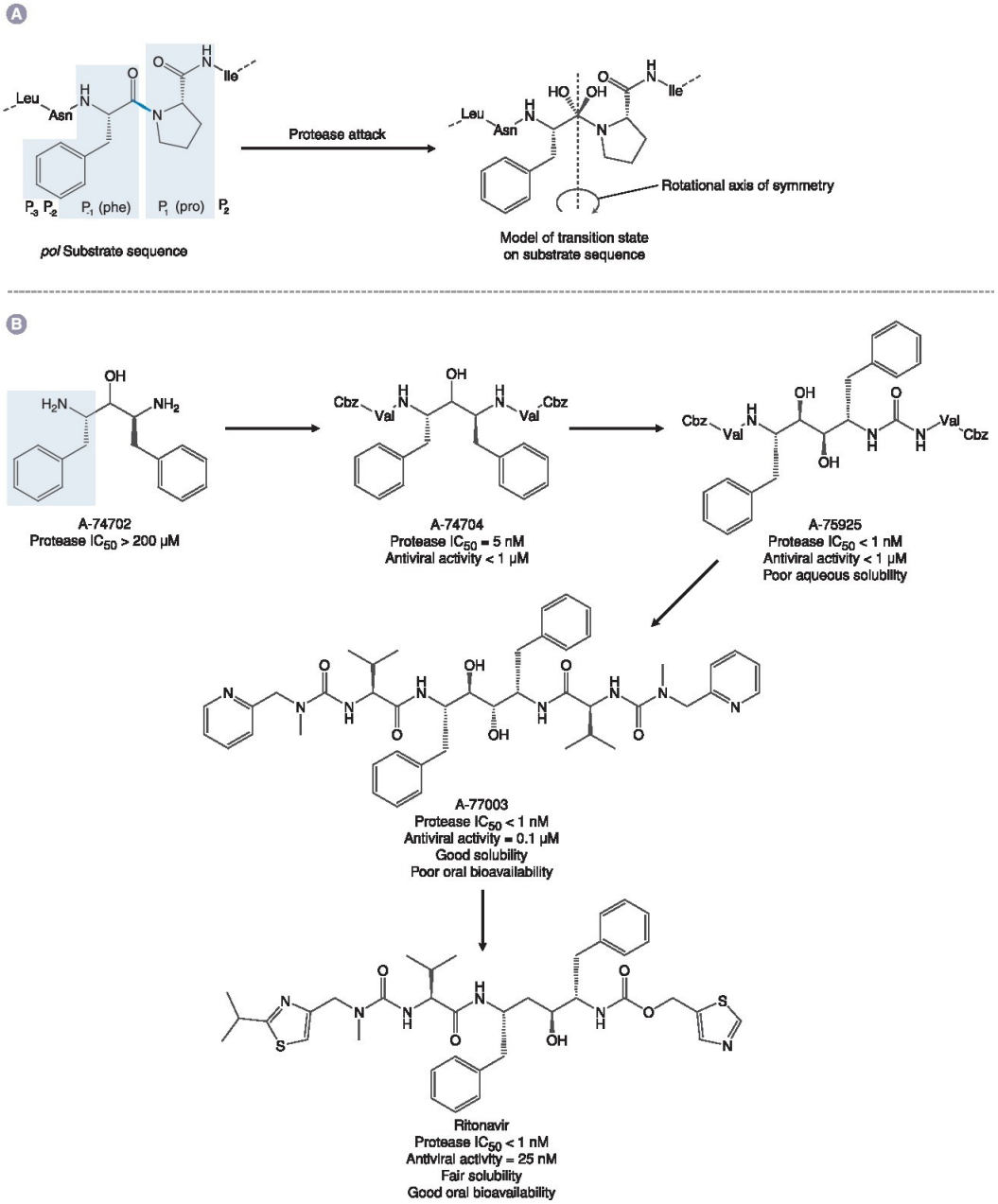

FIGURE 14.14 **Steps in the development of ritonavir**. **A**. On the left is shown a substrate sequence that is cleaved by HIV protease between a phenylalanine (phe) on the N-terminal side of the cleavage (P_{-1}) and a proline (pro) on the C-terminal side (P_1). On the right, the transition state of this sequence during protease cleavage is modeled. The transition state contains a rotational axis of symmetry. **B**. Structure-based development of ritonavir began with A-74072 (*top left*) that mimics the transition state with a single CHOH moiety between two phenylalanines (to maximize symmetry). This compound, which had weak inhibitory activity, was modified by symmetric

addition of other groups (CBZ = carboxybenzyl) to yield A-74074, which was more potent, but was insufficiently soluble. This was modified to mimic the transition state with CHOH groups on each side of the axis of symmetry (A-75925, *top right*). This was also poorly soluble but could be improved by adding more groups (A-77003, *middle*). That compound was not very orally available, so additional modifications, including the removal of a central OH group, were made, resulting in ritonavir (*bottom*). The choices of modifications were aided by crystal structures of intermediate compounds bound to HIV-1 protease. For each compound, the inhibitory potencies against HIV-1 protease *in vitro* (IC_{50}) and against HIV in infected cells (antiviral activity) are provided.

Crystallographic studies reveal that upon binding of protease inhibitors, the enzyme assumes a conformation with closed "flaps" that are unable to undergo conformational changes that help form the active site (Fig. 14.14C; reviewed in Refs.[10,472]). The drugs work in cell culture as expected: HIV-infected cells exposed to protease inhibitors continue to make viral proteins, but these proteins are not processed efficiently.[92] Viral particles bud from the infected cells, but they are immature and noninfectious.[149] Mutations conferring drug resistance map to HIV sequences encoding the protease, confirming that protease is the target.[333]

Mutations in over 30 of the 99 amino acid residues of protease have been selected with the different protease inhibitors, with as many as 24 conferring resistance to any one drug.[459] Some of the mutations affect the active site or sites known to interact with the inhibitors, and thus the mechanism of resistance can be readily understood. However, other mutations affect more distal sites, and it is not clear how they confer resistance, although several lines of evidence raise the possibility that these mutations affect the dynamics of the protease to weaken drug binding ([250] and references therein). Resistance is often cumulative. In some patients, after failing multiple regimens containing protease inhibitors, over 20 of these mutations have accumulated in their virus, demonstrating the remarkable plasticity of this enzyme.[459] There is even an example of a virus whose replication is enhanced by subinhibitory concentrations of a protease inhibitor.[290]

Different protease inhibitors tend to select different mutations, at least initially. Thus, although the drugs all work by similar mechanisms, the first mutation selected for resistance to one protease inhibitor often does not confer resistance to another. This can be understood by considering that although the different drugs tend to share similar functionalities that interact with the protease active site, each drug has a different functionality that confers high affinity binding via interactions with a particular site specific to the drug. Thus, a single mutation that interferes with binding of one drug will not necessarily affect binding of the other. As mutations accumulate, however, cross resistance develops among the protease inhibitors. Mutations affecting protease cleavage sites can also confer resistance.[105] These result in more easily cleaved precursor substrates for the protease. Different protease mutations also have differing impacts on viral fitness. Certain mutations can be readily found in untreated patients, suggesting high fitness, while others are not detected except following drug treatment, and often decrease fitness. Cleavage site mutations can improve the fitness of protease mutants.[105,280,489] These different features of resistance to protease inhibitors have important clinical implications (see section on *HIV therapy*).

Inhibition of Viral Release: Influenza Virus Neuraminidase Inhibitors

Inhibitors of influenza virus neuraminidases block viral release of influenza A and B viruses. The rationale for their action follows from the mechanism of viral attachment. Influenza attaches to cells via interactions between the virion hemagglutinin and sialic acid, which is present on many cellular membrane glycoproteins. However, upon egress of influenza virus from cells at the end of a round of replication, hemagglutinin on nascent virions again binds to sialic acid and tethers virions, preventing release and initiation of new rounds of infection. To overcome this problem, influenza virus encodes a virion neuraminidase, which cleaves sialic acid from the membrane glycoproteins. Studies using influenza virus mutants or an early neuraminidase inhibitor showed that without neuraminidase, the virus remains tethered and cannot spread to other cells.[266,339,340] In 1992, structures of the neuraminidase-sialic acid cleavage product were solved (Fig. 14.15A).[52,448] The structure showed that sialic acid occupied two of three well-formed pockets on the enzyme (Fig. 14.15C). Based largely on this structure, a new sialic acid transition state analog was designed to make energetically favorable interactions in all three of the pockets, principally by adding a guanidino group[452] (Fig. 14.15C). This compound, zanamivir (Fig. 14.15B), inhibits neuraminidase with a Ki of about 0.1 nM. Zanamivir is active against both influenza A and influenza B viruses with potencies of about 30 nM. However, it is not orally bioavailable and must be administered as an aerosol.

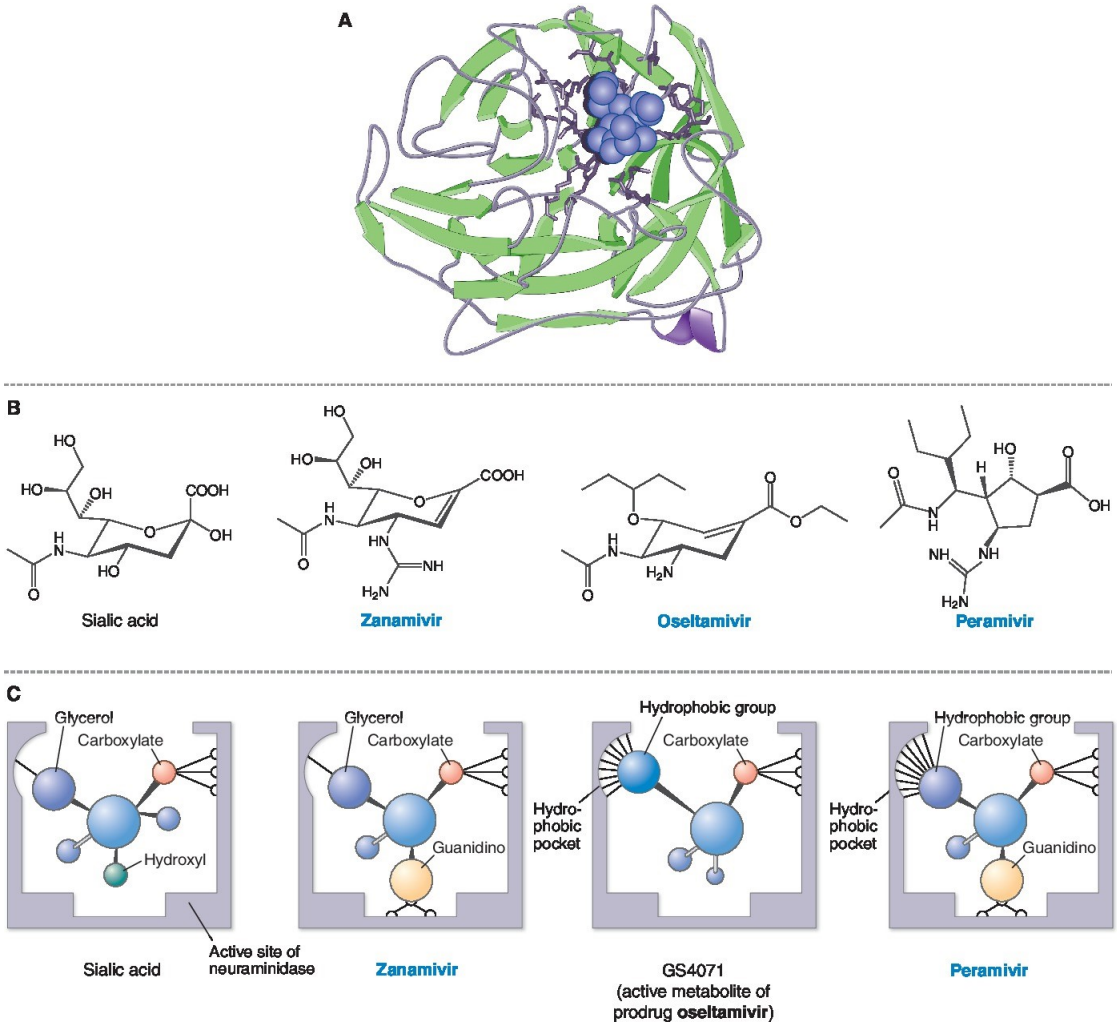

FIGURE 14.15 **Structure-based design of inhibitors of influenza A and B virus neuraminidase**. **A**. Shown is a model of sialic acid (space-filling structure in *dark purple*) bound to the influenza neuraminidase (*green* and *light purple*), with the amino acids that bind sialic acid shown in stick form. This structure was used to design transition state inhibitors that bind with higher affinity than sialic acid. **B**. Structures of sialic acid and the neuraminidase inhibitors oseltamivir, zanamivir, and peramivir. **C**. Diagram of the active site of influenza virus neuraminidase, showing the binding of sialic acid, zanamivir, GS 4071 (oseltamivir is the ethyl ester of this compound), and peramivir showing how the different ligands interact with pockets in the active site. Note that the compounds in **C** are flipped 180° relative to how they are shown in **A** and **B**. (Modified with permission from Li JZ, Coen DM. Pharmacology of viral infections. In: Golan DE, Armstrong EJ, Armstrong AW, eds. *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. 4th ed. Wolters Kluwer; 2017:694-722. **C** is modified from Bryan Christie: Source: R. Scott Rowland, *BioCyst Pharmaceuticals, Inc.*)

Efforts to obtain a neuraminidase inhibitor with better pharmacokinetic properties than zanamivir resulted in oseltamivir (Fig. 14.15B).[217,257] Oseltamivir makes use of a carbocyclic ring based on a transition state analog of sialic acid. Rather than use a guanidino group to occupy a third pocket, it makes stronger contacts with one of the two pockets bound by sialic acid by adding a hydrophobic group (Fig. 14.15C). Oseltamivir's high oral bioavailability depends in part on its being an ester prodrug, which when cleaved gives rise to the active carboxylate (Fig. 14.15B).

Peramivir, which is administered by injection, replaces the 6 membered ring of oseltamivir and zanamivir with a cyclopentane (Fig. 14.15B). Despite the change in core structure, peramivir maintains a similar binding mode for the carboxylate to the other NA inhibitors, as well as occupying the hydrophobic pocket of oseltamivir and the guanidino binding pocket of zanamivir[6,18,486] (Fig. 14.15C). Peramivir has similar NA potency to that observed with zanamivir and oseltamivir.[26,28,165]

Most neuraminidase resistance mutations result in an enzyme that is less inhibited by the drug.[298] As would be expected by the differences in drug binding to the enzyme (Fig. 14.15), mutants resistant to one drug are not necessarily resistant to the other. For example, the H274Y mutation causes a 2-3 order of magnitude decrease in NA IC_{50} for oseltamivir, but a 1-2 order of magnitude decrease in NA affinity for peramivir.[21,165] Still, compared with amantadine, for many years, it was relatively difficult to generate mutants resistant to zanamivir or oseltamivir in the laboratory, and they appeared less frequently in the clinic. This was due in part to most such mutants being less fit than wild-type virus.[203,278] However, fit H275Y viruses have since arisen; their fitness can be attributed to secondary mutations that allow increased expression of the H275Y mutants on the surface of the infected cell.[40,358] As described in the Overview section of this chapter, another class of mutants resistant to the neuraminidase inhibitors contains altered hemagglutinins.

With increasing concerns regarding influenza pandemics, the mechanisms of action and resistance of neuraminidase inhibitors have become very important, as has the development of new anti-influenza drugs such as baloxavir that are active against mutants resistant to neuraminidase inhibitors.

Antiviral Therapies That Target Immune Processes

Two types of drugs approved for treatment of viral infections—imiquimod and interferons—do not inhibit virus replication directly. Rather, they enhance innate host immune responses to viral infection (see Chapter 9). Imiquimod, which is approved for treating certain diseases caused by human papillomaviruses, interacts with Toll-like receptors TLR7 and TLR8 to boost innate immunity, including secretion of interferons.[152,183,208] Interferon alpha is approved for treating HCV, HBV, condyloma acuminata, which is caused by certain human papillomaviruses, and Kaposi sarcoma, which is caused by Kaposi sarcoma–associated herpesvirus (human herpesvirus 8). The actions of interferons are reviewed in Chapter 9. However, which of the many activities induced by interferons contribute to their therapeutic effects (eg, against HCV; reviewed in Ref.[125]) remain poorly understood. Interestingly, genetic polymorphisms in a region encoding certain type III interferons, which are induced by interferon alpha, are associated with response of HCV infections to interferon alpha and ribavirin, with lower expression of these interferons associated with lower responses to therapy.[147,421,433] Follow-up studies of this observation and greater understanding of mechanisms by which viruses inhibit interferon action (eg, HCV; reviewed in Ref.[125]) may facilitate the development of more targeted therapies.

Principles of Antiviral Therapy

General Concepts

Different viruses can have very different dynamics of infection, and these differences have major impacts on the goals and effects of antiviral therapy. Many of these differences relate to the interplay between antiviral therapy and the immune system. Acute viral infections of immunocompetent patients usually are cleared by the immune system in a matter of days. It is not surprising then that antiviral treatment of most such infections in immunocompetent patients reduces the duration of symptoms by only a day or so; as a corollary, treatment must be started as soon as possible to obtain clinical benefits. Indeed, antiviral prophylaxis can be more effective than treatment.

Many of the same considerations apply to recurrent disease caused by reactivation of latent viral infections in immunocompetent patients. In the case of frequently recurring herpes genitalis, suppressive therapy is much more effective than treating each episode. Another issue in acute infections can be the extent to which symptoms are due to viral replication rather than the immune response to the virus. For example, many of the later manifestations of severe COVID-19 are the consequence of the host immune response to SARS-CoV-2 and respond more effectively to treatment with corticosteroids and other immunomodulators than to antivirals.[163,210,366,401,464] Conversely, in the absence of an effective immune response, antiviral therapy may fail to control viral replication, leading to emergence of drug-resistant virus as in the case of HSV, VZV, and HCMV in patients with late-stage AIDS.

Many of the viruses for which antiviral drugs have been developed cause persistent infections. The primary goal in the management of persistent viral infections with antiviral therapy is sustained suppression of viral replication. In contrast with acute infections, treatment of chronic viral infections can be effective even when initiated well after symptoms have appeared. In some cases, such as HCV infections, persistent viral replication occurs in the face of an immune response that usually is incapable of clearing the virus by itself. However, because HCV does not establish latent infection, complete inhibition of viral replication for a sufficient period results in cure. In other cases, such as HCMV reactivation in recipients of stem cell or solid organ transplants, the immune system often cannot control virus replication. Antiviral drugs cannot cure HCMV infections but can suppress viral replication, thereby preventing disease until an effective immune response is restored. The benefits of suppressive prophylactic treatment of HCMV in immunosuppressed patients must be weighed carefully against the toxicities of anti-HCMV drugs and risks of drug resistance (see below).

Drug Resistance in Antiviral Therapy

As summarized earlier in the chapter, the rate at which mutants that are resistant to a given drug will emerge is a function of mutation rate, size of the genomic target for resistance, relative fitness conferred by a particular resistance mutation, replication rate, and size of the preexisting population of virus.[84] All of these factors can have clinical impact. For example, HIV has high mutation and replication rates, and patients have enormous populations of virus by the time they are treated, so resistance can emerge in most patients even with a single codon target whose mutation reduces fitness.[84] By contrast, HSV has lower mutation and replication rates and lower populations of virus, so resistance is less common even with larger target sizes for ACV resistance. In both cases, drug-resistant mutants can recur from latent reservoirs.

Of particular relevance to antiviral drug resistance is the relationship between drug concentrations attained in patients and the suppressive effect on viral replication. At very low levels of drug exposure, minimal viral inhibition is achieved and thus selective pressure favors persistence of wild-type virus. As drug exposure and viral inhibition increase, so too does selection pressure favoring emergence of drug-resistant variants. At higher levels of drug exposure, only variants carrying mutations that confer high-level drug resistance can persist. Achieving complete suppression of viral replication through appropriate drug dosing or use of combination antiviral therapy substantially reduces the risk of drug resistance. For example, combination ART regimens that include the HIV protease inhibitor (PI) atazanavir select for the I50L substitution in HIV protease, which confers modest levels of resistance.[88] By contrast, when atazanavir is coadministered with a low dose of ritonavir, an HIV PI that potently inhibits hepatic drug metabolism, thereby leading to sustained high levels of atazanavir, resistance is rarely observed.[253] The ultimate goal of antiviral therapy, especially for viruses like HIV, HBV, and HCV, which have high mutation rates, high replication rates, and high preexisting populations, is to apply drug regimens that completely inhibit virus replication.

Clinical Impact of Drug Resistance

It may seem obvious that drug resistance is important clinically. However, many treatment failures are not due to drug resistance, and many drug-resistant viruses do not cause treatment failures. For acute infections like influenza, which are largely controlled by the immune system, antiviral drug resistance is generally not a major problem for the patient in whom it arises. Therefore, during monotherapy with neuraminidase inhibitors, variants probably emerge in all patients within a week, but in immunocompetent patients, that is when the infection has largely resolved. Transmission of resistant virus to others can occur, however, and the antiviral efficacy will be impaired in these secondary cases. By contrast, in persistent infections, accumulation of resistance mutations progressively reduces the efficacy of antiviral regimens. Continued replication in the presence of drug selects for even greater levels of resistance to each administered drug and progressive cross resistance to

drugs of the same class. These factors drive a vicious cycle of treatment failure and yet more difficult treatment challenges.

Drug-resistant herpesviruses and HIV can establish latent infections. Drug-resistant HIV, HBV, and influenza A viruses have been transmitted to other individuals.[117,139,297] In the case of HIV, resistant virus in blood or genital secretions can be transmitted during sex, needle sharing, or childbirth.[263] In the United States, one or more transmitted drug resistance mutation was detected in HIV from nearly 20% of newly diagnosed persons during the period 2014-2018.[291] Antiretroviral drug resistance in lower- and middle-income countries has become increasingly appreciated with the rollout of treatment access.[476]

Strategies to Combat Drug Resistance

The increasing prevalence of antiviral drug resistance raises challenges to the effective treatment of individuals and to the public health similar to those that have resulted from widespread antibiotic resistance. One strategy to combat this problem is to test viruses for drug resistance before choosing antiviral regimens. Resistance testing can help determine which drugs will not work (thereby diminishing cost, toxicity, and inconvenience) and which drugs are most likely to be effective. These tests can be performed most rapidly if they are genotypic (ie, detecting mutations at the level of DNA or RNA) rather than phenotypic (ie, measuring changes in drug susceptibility). Because the turn-around time for obtaining resistance test results may delay treatment initiation by days to a few weeks, resistance testing for individual patients is more feasible for chronic viral infections (eg, HIV) than for acute infections (eg, influenza). For this reason, active surveillance to assess the prevalence of resistance to first-line drugs among circulating viruses is essential.

A second potential strategy is combination chemotherapy. This strategy combats resistance on several levels. First, the probability of a virus being resistant to multiple different drugs (with different mutations conferring resistance) is the product of the probabilities of resistance to each drug. This approach makes it much less likely that a preexisting virus in the patient will be resistant to all of the drugs. Second, the combination is likely to suppress replication more completely than would any single drug alone, thereby reducing the chance for resistance to develop. Third, members of the combination might synergize, thereby providing even greater efficacy. It is crucial that drugs in the combination not antagonize each other's activities by mechanism of action or pharmacologic interaction. Fourth, a mutation conferring resistance to one drug in a combination might yield clinical advantages, for example, by making the virus less fit or by sensitizing otherwise resistant viruses to a second drug. Such advantages have been invoked for the M184V mutation in HIV and the M204V/I mutation in HBV that confer resistance to 3TC and FTC.[100,232]

Drug resistance is relatively uncommon with certain virus infections, such as herpesvirus infections in immunocompetent persons, which, together with the various downsides of adding additional drugs, explains why combination chemotherapy is rarely used for treating herpesvirus or influenza virus infections.

HIV Therapy

Since AZT was first approved for clinical use, more than 30 drugs or drug combinations have been approved for treating HIV (Fig. 14.16). This remarkable drug development experience helped elucidate many of the principles of antiviral therapy described above. A review of the clinical evaluation of each of the anti-HIV drugs and each of the combinations of these drugs for patients in various stages of disease is beyond the scope of this chapter. Guidelines summarizing the practical use of currently available antiretrovirals are frequently updated.[343,383]

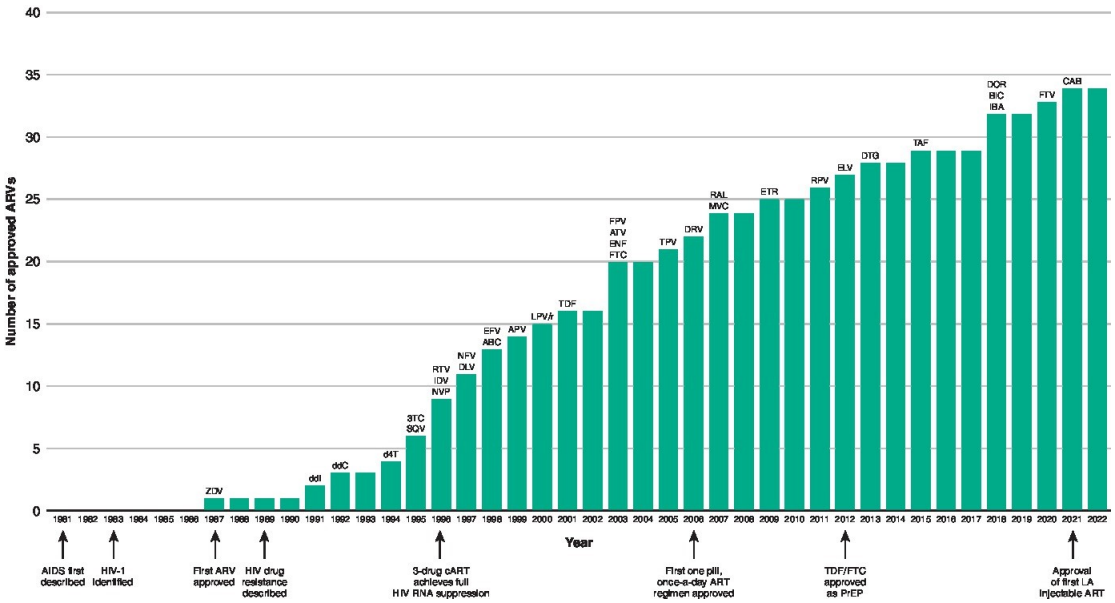

FIGURE 14.16 **Time line of AIDS therapies**. The dates of important events in the understanding and therapy of AIDS are provided together with the dates of FDA approval of antiretroviral drugs. The bars represent the progressive accrual of approved drugs. Abbreviations: ZDV, zidovudine (AZT); ddI, didanosine; ddC, zalcitabine; d4T, stavudine; 3TC, lamivudine; SQV, saquinavir; RTV, ritonavir; IDV, indinavir; NVP, nevirapine; NFV, nelfinavir; DLV, delavirdine; EFV, efavirenz; ABC, abacavir; APV, amprenavir; LPV/r; lopinavir/ritonavir; TDF, tenofovir; FPV, fosamprenavir; ATV, atazanavir; ENF, enfuvirtide; FTC, emtricitabine; TPV, tipranavir; DRV, darunavir; RAL, raltegravir; MVC, maraviroc; ETV, etravirine; RIL, rilpivirine; ELV, elvitegravir; DTG, dolutegravir; TAF, tenofovir alafenamide; DOR, doravirine; BIC, bictegravir; IBA, ibalizumab; CAB, cabotegravir; ARV, antiretroviral; cART, combination antiretroviral therapy; PrEP, preexposure prophylaxis; LA, long acting. After this figure was completed, lenacapavir was approved (late 2022). (Adapted from a figure created by Douglas R. Richman.)

A large body of data from randomized controlled trials and longitudinal cohort studies informs treatment guidelines developed by various groups. The general principles are clear: to be effective, treatment must be sufficiently potent to suppress plasma viremia to below the limits of detection of sensitive assays and must be sufficiently simple and well tolerated that patients will be capable of adhering to the prescribed regimen with a minimum of missed doses. Because current therapy is incapable of eradicating HIV-1 infection, once such therapy is started, most patients will require it lifelong.

Since 2016, the World Health Organization HIV treatment guidelines have recommended initiation of ART for all persons with HIV infection regardless of CD4 count or stage of HIV infection. As of this writing (2022), regimens preferred by the U.S. Department of Health and Human Services *Guidelines for Use of Antiretroviral Agents in Adults and Adolescents with HIV* include an integrase strand transfer inhibitor (INSTI; either bictegravir or dolutegravir) in combination with tenofovir plus FTC or 3TC.[121,343,383,477] The approval of long-acting formulations of the INSTI cabotegravir plus the NNRTI rilpivirine as monthly or bimonthly injectable agents provides an alternative to daily oral therapy in appropriate patients.[331,334,428] Newer drugs in novel classes such as ibalizumab and fostemsavir generally are reserved for highly treatment-experienced patients with limited treatment options due to multidrug-resistant HIV.[115,226]

Therapy of Herpesviruses

HSV and VZV Therapies

ACV, the mechanism of which is detailed in this chapter, set the standard for the development of antiviral drugs. Randomized, controlled trials demonstrated efficacy of the intravenous drug for serious systemic infections such as herpes encephalitis, neonatal herpes, and disseminated varicella zoster in immunocompromised hosts.[463] The oral formulation, which has negligible toxicity, is used widely for the treatment of primary and recurrent HSV-1 and HSV-2, the prophylaxis of recurrent HSV, and the treatment of uncomplicated VZV. The earlier in the presentation of symptoms, the more effective the treatment, and prophylaxis is more dependable than treatment. The appearance of resistant isolates has been remarkably rare in immunocompetent patients and occurs in a minority of immunocompromised patients. ACV-resistant HSV and VZV did become a significant challenge in patients with AIDS before the availability of combination antiretroviral therapy.[120,337] The expanded number of resistant infections reflected the number of patients with AIDS, the prevalence of recurrent HSV and VZV infection in these patients, and the duration and magnitude of these opportunistic infections because of the severely reduced antiviral immunity in these hosts. Foscarnet shows activity against some ACV-resistant infections,[384] but resistance to this DNA polymerase inhibitor has developed as well.[385]

Two limitations of oral ACV have been its limited oral bioavailability (~15%) and short half-life. These limitations require administration of large pills as often as every 4 hours. As described earlier in this chapter, valacyclovir and famciclovir overcome these limitations. Both valacyclovir and famciclovir can be used in place of oral ACV. In most situations, valacyclovir is preferred due to dosing convenience. In addition, once-daily administration of the ACV prodrug valacyclovir reduces the risk of transmission of genital HSV.[90] Because of the more prolonged maintenance of higher levels of antiviral activity, valacyclovir and famciclovir are also used in place of intravenous or high-dose oral ACV for the treatment of uncomplicated herpes zoster in the immunocompromised host.[35,445]

HCMV Therapies

As reviewed earlier in the chapter, the lack of potency of ACV against HCMV led to the development of ganciclovir. Ganciclovir is more toxic than ACV. However, it is the first-line therapy for HCMV disease in the immunocompromised host. The eventual development of valganciclovir has now provided an orally bioavailable drug for HCMV that can achieve levels approaching those of intravenous ganciclovir without the cost, inconvenience, and complications of intravenous dosing.

Although ganciclovir and valganciclovir initially were used for the treatment of retinitis and other end-organ disease due to HCMV in patients with HIV, the incidence of these infections has diminished drastically since the introduction of effective combination ART. Consequently, use of ganciclovir and other anti-HCMV agents is now directed principally to the prevention and treatment of HCMV disease in recipients of stem cell and solid organ transplants. In the setting of stem cell transplantation, preventive therapy may be preemptive (guided by the level of HCMV viremia as measured by quantitative PCR) or prophylactic (given in the immediate posttransplant period to high-risk patients regardless of HCMV viremia). Previously, the benefits of prophylaxis with ganciclovir or valganciclovir needed to be balanced against the risk of prolonged neutropenia, the principal toxicity of these agents. The approval of letermovir for HCMV prophylaxis in this setting is changing practice, given the absence of myelotoxicity or nephrotoxicity associated with this drug.[287]

Foscarnet and cidofovir are also approved for the treatment of HCMV. Although equally effective, foscarnet is used only for ganciclovir-resistant HCMV infections because of inconvenience and nephrotoxicity.[439] Cidofovir also is used for the treatment of ganciclovir-resistant HCMV. It has the advantage of requiring administration only once every 1 or 2 weeks due to its very prolonged intracellular half-life but shares with foscarnet the disadvantage of renal toxicity. Maribavir is approved for treatment of HCMV infections in transplant recipients who fail to respond to other available antiviral agents. Although a phase 3, double-blind, randomized controlled trial failed to show a benefit of maribavir with respect to incidence of CMV disease,[288] a subsequent open-label phase 3 trial that used a higher dose of maribavir in patients with HCMV refractory to treatment with ganciclovir (or valganciclovir), foscarnet, or cidofovir demonstrated that a significantly greater proportion of participants achieved clearance of HCMV viremia in the maribavir arm as compared to the investigator-assigned treatment arm.[16]

Antiviral therapy has not yet been shown in randomized trials to significantly alter disease attributable to the other human herpesviruses including Epstein-Barr virus (EBV) and human herpesvirus 6 (HHV6), HHV7, and HHV8. Retrospective analyses and case reports suggest a possible clinical benefit of treating HHV6 encephalitis in the setting of stem cell transplantation with foscarnet, ganciclovir, or cidofovir, each of which shows activity against HHV6 *in vitro*,[187] but even when started promptly survivors may suffer significant neurologic sequelae. No available antiherpesvirus drug effectively addresses the problem of viral latency.

Therapy of Viral Hepatitis

HBV Therapy

The complex natural history of HBV infection is described elsewhere in this text. Criteria based on disease stage and level of HBV viremia, and the availability of more effective drugs, have increased the treatment indications and the long-term success of therapy.[122,475] An important complication is that chronic HBV infection implies that immune responses toward the virus are impaired. In addition, chronic HBV entails the existence of large numbers of cells containing covalently closed circular HBV DNA, a situation akin to herpesvirus latency. For all practical purposes, this factor may preclude eradication of HBV infection. In this context, for therapy to be successful, the immune response must be restored, chronic antiviral suppression must be maintained, or both.

A significant proportion, but not all, carriers of HBV progress to cirrhosis, hepatic failure, or hepatocellular carcinoma. Antiviral therapy has been shown to prevent the development of these complications and, in fact, to reverse cirrhotic histopathology.[424] Whom, when, and how to treat are decisions based on risk of progression and therapeutic index of the various treatments.[122] Treatment efficacy is affected by the regimen used as well as viral genotype, disease and virologic status, age, and comorbidities such as HIV coinfection. Because treatment interruption can lead to a serious flare of acute hepatitis, once initiated anti-HBV treatment generally must be continued lifelong.

Five nucleoside analogs have been approved for the treatment of hepatitis B. The experiences with these drugs recapitulate many of the experiences of HIV therapy. Whereas 3TC, telbivudine, and adefovir are effective at reducing plasma HBV DNA, continued use is associated with increasing proportions of patients with resistant mutants who experience treatment failure.[270] Entecavir and tenofovir (formulated as one of two prodrugs) have displaced other drugs for HBV infection because of their high rates of virus suppression with monotherapy and the low rates of emergence of drug-resistant virus during treatment.[122,406,438,475] The combination of tenofovir and emtricitabine, which is active against HBV, has been approved for the treatment of HIV and is used routinely in patients who are coinfected with HIV and HBV.

HCV Therapy

A critical difference between HBV (or HIV) and HCV is that HCV has no DNA intermediate that precludes eradication; hence, HCV is curable. Whereas treatment previously depended on the combination of IFN-α plus ribavirin, such treatment has been supplanted entirely by combination therapy with orally bioavailable small molecule antivirals targeting the NS3/4A protease, NS5A, or the NS5B RNA polymerase (so-called “directly acting” antiviral drugs) that have revolutionized the treatment of HCV. Regimens including various combinations of these drugs are able to effect cure (as assessed by sustained virologic suppression) in nearly all patients with HCV after just 8-12 weeks of treatment. The choice of regimen depends on presence or absence of cirrhosis, HCV genotype, and whether or not the patient has received prior treatment for HCV. Specific guidance from the American Association for the Study of Liver Diseases and the Infectious Diseases Society of America can be found at www.hcvguidelines.org. The combination of the NS3/NS4A protease inhibitor glecaprevir plus the NS5A inhibitor pibrentasvir administered daily for 8 weeks is recommended for patients with or without compensated cirrhosis regardless of genotype[14,15,130,233,490]; the combination of NS5B RNA polymerase inhibitor sofosbuvir plus the NS5A inhibitor velpatasvir administered daily for 12 weeks can also be used,[126,131] but addition of voxilaprevir or weight-based dosing of ribavirin is necessary in patients with infection due to HCV genotype 3 carrying the NS5A inhibitor resistance–associated mutation Y93H.[45,46,204,351] Regimens including combinations of ledipasvir plus sofosbuvir[3,44] or elbasvir plus grazoprevir[132,491] may also be useful in some patients depending on HCV genotype.

Influenza Therapy

Treatment of influenza relies on the neuraminidase inhibitors (oseltamivir, peramivir, and zanamivir) and the cap-dependent endonuclease inhibitor baloxavir. Each of these drugs is effective in treating both influenza A and B[178,179,221,315,321,359,441]; oseltamivir, zanamivir, and baloxavir are also effective for postexposure prophylaxis.[175,177,201] Oseltamivir and baloxavir are dosed orally, whereas zanamivir must be administered by inhalation and peramivir is available only for intravenous administration. Although the adamantanes amantadine and rimantadine were the first antiviral drugs used for prevention of influenza A, because of widespread resistance to these M2 inhibitors, they no longer are recommended for use.

Because of its oral bioavailability, oseltamivir is preferred among the neuraminidase inhibitors for treatment and prevention of influenza, except when a high proportion of circulating influenza A strains are oseltamivir resistant. The inhalational administration of zanamivir risks uneven distribution in the respiratory tract and occasionally bronchospasm. Baloxavir has the advantage of requiring only a single dose and has greater activity against influenza B as compared to oseltamivir.[179] Although baloxavir retains activity against influenza A strains resistant to neuraminidase inhibitors, emergence of I38T, M, or F substitutions in the polymerase PA subunit that confer resistance to baloxavir occurred in 9.7% of participants assigned to baloxavir in a phase 3 trial.[179] For this reason, oseltamivir generally is preferred over baloxavir. In theory, a combination of oseltamivir and baloxavir might prevent emergence of resistance in influenza A while providing optimal activity against influenza B, but limited clinical trials conducted to date have not shown improved outcomes.[230] Because experience with baloxavir in patients with severe influenza is limited, oseltamivir is preferred in this setting as well. Use of peramivir typically is reserved for patients unable to take oral therapy. An intravenous formulation of zanamivir is available in the United Kingdom and Europe Union, but as of this writing is not approved for use in the United States.[405]

High-level resistance due to His to Tyr mutation in codon 275 of the N1 neuraminidase has emerged during treatment of H1N1 in children and in patients treated for H5N1 infection.[95,377] This mutation confers cross resistance to peramivir but not to zanamivir.[164,320,461] By itself, the H275Y (H274Y in N2) mutation reduces fitness *in vitro* and *in vivo*,[203] although these mutants are transmissible to ferrets.[184] However, as reviewed earlier in the section on neuraminidase inhibitor mechanisms, oseltamivir-resistant H1N1 viruses containing H275Y together with secondary mutations that increase fitness emerged during the 2007-2008 influenza season and became the predominant influenza A strain circulating in 2008-2009[98,176]; those oseltamivir-resistant viruses showed no loss of fitness, transmissibility, or pathogenicity.[297] In that setting, zanamivir was recommended as the preferred antiviral for treatment and prevention of influenza A (baloxavir was not yet available). Fortunately, the prevalence of oseltamivir resistance has been infrequent in subsequent influenza seasons.

SARS-CoV-2 Therapy

The explosion of the SARS-CoV-2 pandemic in early 2020 prompted an urgent search for antiviral drugs that might prevent disease progression and reduce mortality of COVID-19. Two years into the pandemic, three agents have emerged as small-molecule inhibitors of SARS-CoV-2 with demonstrated clinical efficacy, including remdesivir, nirmatrelvir, and molnupiravir. These agents have broad activity against SARS-CoV-1,

MERS, and SARS-CoV-2. In contrast to mAbs, these drugs retain potency against a wide range of SARS-CoV-2 variants.[430,447] As with treatment for influenza, antiviral therapy for SARS-CoV-2 has the greatest impact when initiated as soon as possible after symptom onset.

The RdRp inhibitor remdesivir is approved for treatment of persons with SARS-CoV-2 infection. Clinical trials have shown its effectiveness in preventing disease progression among outpatients with symptomatic SARS-CoV-2 and risk factors for severe COVID-19, and in shortening the time to recovery among patients with COVID-19 and evidence of lower respiratory tract infection.[30,409] Although comparison with contemporaneous control groups suggest a survival benefit for hospitalized patients treated with remdesivir, randomized, controlled clinical trials have failed to demonstrate a reduction in mortality.[1,312,328,329,464] When administered to symptomatic outpatients at risk for severe COVID-19, remdesivir reduces the risk of COVID-19–related hospitalization or death by 87%.[157] The need to administer remdesivir intravenously has proven to be a logistical barrier to its use in the outpatient setting. Although resistance to remdesivir can be selected by serial passage *in vitro*, to date clinical use has not been accompanied by emergence of resistance.[129,413,429]

Nirmatrelvir, an orally bioavailable inhibitor of the chymotrypsinlike (CL) "main" protease of SARS-CoV-2, is highly effective in preventing disease progression among outpatients with symptomatic infection who are at risk for progression to severe COVID-19.[349] To achieve adequate plasma concentrations, nirmatrelvir is coadministered with a low dose of the cytochrome P450 3A/4 inhibitor ritonavir (an HIV protease inhibitor). Inclusion of ritonavir with nirmatrelvir potentially limits its use due to interactions with certain drugs that eligible patients may require for coexisting medical conditions (eg, clopidogrel; amiodarone; rifampin).[289] The nucleoside analog molnupiravir has more modest activity, reducing the risk of hospitalization or death by 31%.[205] It is approved for the treatment of confirmed SARS-CoV-2 infection in adults who are at high risk for progression to severe COVID-19 for whom alternative COVID-19 treatment options are not accessible or clinically appropriate, but its properties as a teratogen limit use among women of child-bearing potential.

Poxvirus Therapy

Although smallpox has been eradicated as a human disease, the continued maintenance of stocks of variola virus leave open the possibility of renewed outbreaks of smallpox, resulting either from accidental laboratory transmission or, more ominously, due to purposeful release as an act of bioterrorism or biological warfare. Two agents, tecovirimat and brincidofovir, have been approved for use in the treatment of smallpox.[57,453] Both drugs were approved under the FDA's "Animal Rule," which allows approval of drugs for human use based on results of appropriate animal trials when clinical trials in humans cannot be conducted.[162] Given the nature of their approval and the intended circumstances for their use, tecovirimat and brincidofovir are available only through the United States Government's Strategic National Stockpile. Although approved for variola, both drugs have been used anecdotally for treatment of severe complications of vaccinia administration (smallpox vaccination). Data from animal models suggest that tecovirimat and brincidofovir would also be useful for treating mpox (formerly known as monkeypox); indeed, tecovirimat proved to be highly effective in treatment of mpox during the 2022 outbreak.[196,382]

Conclusion and Future Directions

Antiviral drugs ameliorate viral disease and save lives. Understanding viral replication and virus-host interactions is crucial for understanding the mechanisms of antiviral agents and for the discovery of new ones. Likewise, antiviral agents provide excellent tools for investigating viral biology and biochemistry and virus-host interactions. Despite the remarkable progress in antiviral therapeutics made since publication of the previous edition of this textbook, effective treatment is available for only a small number of the clinically important viruses discussed in subsequent chapters. Viruses for which treatment is needed include respiratory syncytial virus, adenovirus, enteroviruses, flaviviruses other than HCV, alphaviruses, and rabies virus, to name a few. There remains a considerable unmet medical need that can be served by antiviral drugs, and we continue to learn how to use them.

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REFERENCES

1. Ader F, Bouscambert-Duchamp M, Hites M, et al. Remdesivir plus standard of care versus standard of care alone for the treatment of patients admitted to hospital with COVID-19 (DisCoVeRy): a phase 3, randomised, controlled, open-label trial. Lancet Infect Dis. 2022;22:209-221.

2. Aduma PP, Connelly MC, Srinivas RV, Fridland A. Metabolic diversity and antiviral activities of acyclic nucleoside phosphonates. Mol Pharmacol. 1995;47:816-822.

3. Afdhal N, Reddy KR, Nelson DR, et al. Ledipasvir and sofosbuvir for previously treated HCV genotype 1 infection. N Engl J Med. 2014;370:1483-1493.

4. Agostini ML, Andres EL, Sims AC, et al. Coronavirus susceptibility to the antiviral remdesivir (GS-5734) is mediated by the viral polymerase and the proofreading exoribonuclease. MBio. 2018;9:e00221-18.

5. Agostini ML, Pruijssers AJ, Chappell JD, et al. Small-molecule antiviral beta-d-N (4)-hydroxycytidine inhibits a proofreading-intact coronavirus with a high genetic barrier to resistance. J Virol. 2019;93:e01348-19.

6. Alame MM, Massaad E, Zaraket H. Peramivir: a novel intravenous neuraminidase inhibitor for treatment of acute influenza infections. Front Microbiol. 2016;7:450.

7. Alian A, Griner SL, Chiang V, et al. Catalytically-active complex of HIV-1 integrase with a viral DNA substrate binds anti-integrase drugs. Proc Natl Acad Sci U S A. 2009;106: 8192-8197.

8. Anastassopoulou CG, Marozsan AJ, Matet A, et al. Escape of HIV-1 from a small molecule CCR5 inhibitor is not associated with a fitness loss. PLoS Pathog. 2007;3:e79.

9. Andrei G, Gammon DB, Fiten P, et al. Cidofovir resistance in vaccinia virus is linked to diminished virulence in mice. J Virol. 2006;80:9391-9401.

10. Appelt K. Crystal structures of HIV-1 protease-inhibitor complexes. Perspect Drug Discov Des. 1993;1:23-48.

11. Appleby TC, Perry JK, Murakami E, et al. Viral replication. Structural basis for RNA replication by the hepatitis C virus polymerase. Science. 2015;347:771-775.

12. Arion D, Kaushik N, McCormick S, Borkow G, Parniak MA. Phenotypic mechanism of HIV-1 resistance to 3′-azido-3′-deoxythymidine (AZT): increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase. Biochemistry. 1998;37:15908-15917.

13. Ascher DB, Wielens J, Nero TL, Doughty L, Morton CJ, Parker MW. Potent hepatitis C inhibitors bind directly to NS5A and reduce its affinity for RNA. Sci Rep. 2014;4:4765.

14. Asselah T, Kowdley KV, Zadeikis N, et al. Efficacy of glecaprevir/pibrentasvir for 8 or 12 weeks in patients with hepatitis C virus genotype 2, 4, 5, or 6 infection without cirrhosis. Clin Gastroenterol Hepatol. 2018;16:417-426.

15. Asselah T, Lee SS, Yao BB, et al. Efficacy and safety of glecaprevir/pibrentasvir in patients with chronic hepatitis C virus genotype 5 or 6 infection (ENDURANCE-5,6): an open-label, multicentre, phase 3b trial. Lancet Gastroenterol Hepatol. 2019;4:45-51.

16. Avery RK, Alain S, Alexander BD, et al. Maribavir for refractory cytomegalovirus infections with or without resistance post-transplant: results from a phase 3 randomized clinical trial. Clin Infect Dis. 2022;75(4):690-701.

17. Baba M, Nishimura O, Kanzaki N, et al. A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. Proc Natl Acad Sci U S A. 1999;96:5698-5703.

18. Babu YS, Chand P, Bantia S, et al. BCX-1812 (RWJ-270201): discovery of a novel, highly potent, orally active, and selective influenza neuraminidase inhibitor through structure-based drug design. J Med Chem. 2000;43:3482-3486.

19. Back M, Johansson PO, Wangsell F, et al. Novel potent macrocyclic inhibitors of the hepatitis C virus NS3 protease: use of cyclopentane and cyclopentene P2-motifs. Bioorg Med Chem. 2007;15:7184-7202.

20. Baek S-H, Kwak J-Y, Lee SH, et al. Lipase activities of p37, the major envelope protein of vaccinia virus. J Biol Chem. 1997;272:32042-32049.

21. Baek YH, Song MS, Lee EY, et al. Profiling and characterization of influenza virus N1 strains potentially resistant to multiple neuraminidase inhibitors. J Virol. 2015;89: 287-299.

22. Bailey TR, Rippin SR, Opsitnick E, et al. N-(3,3a,4,4a,5,5a,6,6a-Octahydro-1,3-dioxo-4,6- ethenocycloprop[f]isoindol-2-(1H)-yl)carboxamides: identification of novel orthopoxvirus egress inhibitors. J Med Chem. 2007;50:1442-1444.

23. Baker JD, Uhrich RL, Kraemer GC, Love JE, Kraemer BC. A drug repurposing screen identifies hepatitis C antivirals as inhibitors of the SARS-CoV2 main protease. PLoS One. 2021;16:e0245962.

24. Baldanti F, Lurain N, Gerna G. Clinical and biologic aspects of human cytomegalovirus resistance to antiviral drugs. Hum Immunol. 2004;65:403-409.

25. Baldanti F, Underwood MR, Stanat SC, et al. Single amino acid changes in the DNA polymerase confer foscarnet resistance and slow-growth phenotype, while mutations in the UL97-encoded phosphotransferase confer ganciclovir resistance in three double resistant human cytomegalovirus strains recovered from patients with AIDS. J Virol. 1996;70:1390-1395.

26. Bantia S, Parker CD, Ananth SL, et al. Comparison of the anti-influenza virus activity of RWJ-270201 with those of oseltamivir and zanamivir. Antimicrob Agents Chemother. 2001;45:1162-1167.

27. Barrera A, Guerra B, Notvall L, Lanford RE. Mapping of the hepatitis B virus pre-S1 domain involved in receptor recognition. J Virol. 2005;79:9786-9798.

28. Barrett S, Mohr PG, Schmidt PM, McKimm-Breschkin JL. Real time enzyme inhibition assays provide insights into differences in binding of neuraminidase inhibitors to wild type and mutant influenza viruses. PLoS One. 2011;6:e23627.

29. Bean WJ, Threlkeld SC, Webster RG. Biologic potential of amantadine resistant influenza A virus in an avian model. J Infect Dis. 1989;159:1050-1056.

30. Beigel JH, Tomashek KM, Dodd LE, et al. Remdesivir for the treatment of covid-19—final report. N Engl J Med. 2020;383:1813-1826.

31. Belema M, Meanwell NA. Discovery of daclatasvir, a pan-genotypic hepatitis C virus NS5A replication complex inhibitor with potent clinical effect. J Med Chem. 2014;57:5057-5071.

32. Belshe RB, Hall Smith M, Hall CB, Betts R, Hay AJ. Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. J Virol. 1988;62:1508-1512.

33. Berger C, Romero-Brey I, Radujkovic D, et al. Daclatasvir-like inhibitors of NS5A block early biogenesis of hepatitis C virus-induced membranous replication factories, independent of RNA replication. Gastroenterology. 2014;147:1094-1105 e25.

34. Bester SM, Wei G, Zhao H, et al. Structural and mechanistic bases for a potent HIV-1 capsid inhibitor. Science. 2020;370:360-364.

35. Beutner KR, Friedman DJ, Forszpaniak C, Andersen PL, Wood MJ. Valciclovir compared with acyclovir for improved therapy for herpes

zoster in immunocompetent adults. Antimicrob Agents Chemother. 1995;39:1546-1553.

36. Bigalke JM, Heldwein EE. Nuclear exodus: herpesviruses lead the way. Annu Rev Virol. 2016;3:387-409.

37. Biron KK, Harvey RJ, Chamberlain SC, et al. Potent and selective inhibition of human cytomegalovirus replication by 1263W94, a benzimidazole L-riboside with a unique mode of action. Antimicrob Agents Chemother. 2002;46:2365-2372.

38. Biron KK, Stanat SC, Sorrell JB, et al. Metabolic activation of the nucleoside analog 9-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]guanine in human diploid fibroblasts infected with human cytomegalovirus. Proc Natl Acad Sci U S A. 1985;82:2473-2477.

39. Blasco R, Moss B. Extracellular vaccinia virus formation and cell-to-cell transmission are prevented by deletion of the gene encoding the 37.000-dalton outer envelope protein. J Virol. 1991;65:5910-5920.

40. Bloom JD, Gong LI, Baltimore D. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. Science. 2010;328:1272-1275.

41. Boeckh M, Englund J, Li Y, et al. Randomized controlled multicenter trial of aerosolized ribavirin for respiratory syncytial virus upper respiratory tract infection in hematopoietic cell transplant recipients. Clin Infect Dis. 2007;44:245-249.

42. Bogner E, Radsak K, Stinski MF. The gene product of human cytomegalovirus open reading frame UL56 binds the pac motif and has specific nuclease activity. J Virol. 1998;72:2259-2264.

43. Boucher CAB, O'Sullivan E, Mulder JW, et al. Ordered appearance of zidovudine resistance mutations during treatment of 18 human immunodeficiency virus-positive subjects. J Infect Dis. 1992;165:105-110.

44. Bourliere M, Adhoute X, Ansaldi C, et al. Sofosbuvir plus ledipasvir in combination for the treatment of hepatitis C infection. Expert Rev Gastroenterol Hepatol. 2015;9:1483-1494.

45. Bourliere M, Gordon SC, Flamm SL, et al. Sofosbuvir, velpatasvir, and voxilaprevir for previously treated HCV infection. N Engl J Med. 2017;376:2134-2146.

46. Bourliere M, Gordon SC, Schiff ER, et al. Deferred treatment with sofosbuvir-velpatasvir-voxilaprevir for patients with chronic hepatitis C virus who were previously treated with an NS5A inhibitor: an open-label substudy of POLARIS-1. Lancet Gastroenterol Hepatol. 2018;3:559-565.

47. Bozzette SA, McCutchan JA, Spector SA, Wright B, Richman DD. A cross-sectional comparison of persons with syncytium- and non-syncyticum-inducing human immunodeficiency virus. J Infect Dis. 1993;168:1993.

48. Brown DG, Visse R, Sandhu G, et al. Crystal structures of the thymidine kinase from herpes simplex virus type-1 in complex with deoxythymidine and ganciclovir. Nat Struct Biol. 1995;2:876-880.

49. Bryk P, Brewer MG, Ward BM. Vaccinia virus phospholipase protein F13 promotes rapid entry of extracellular virions into cells. J Virol. 2018;92:e02154-17.

50. Bui M, Whttaker G, Helenius A. Effect of M1 protein and low pH on nuclear transport of influenza virus ribonucleoproteins. J Virol. 1996;70:8391-8401.

51. Bukrinskaya AG, Vorkunova NK, Kornilayeva GV, Narmanatova RA, Vorkunova GK. Influenza virus uncoating in infected cells and effect of rimantadine. J Gen Virol. 1982;60:49-59.

52. Burmeister WP, Ruigrok RW, Cusack S. The 2.2.Å resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. EMBO J. 1992;11:49-56.

53. Cady SD, Schmidt-Rohr K, Wang J, Soto CS, Degrado WF, Hong M. Structure of the amantadine binding site of influenza M2 proton channels in lipid bilayers. Nature. 2010;463:689-692.

54. Cammack N, Rouse P, Marr CL, et al. Cellular metabolism of (-) enantiomeric 2′-deoxy-3′-thiacytidine. Biochem Pharmacol. 1992;43:2059-2064.

55. Cardozo EF, Ji D, Lau G, et al. Disentangling the lifespans of hepatitis C virus-infected cells and intracellular vRNA replication-complexes during direct-acting anti-viral therapy. J Viral Hepat. 2020;27:261-269.

56. Carrington M, Dean M, Martin MP, O'Brien SJ. Genetics of HIV-1 infection: chemokine receptor CCR5 polymorphism and its consequences. Hum Mol Genet. 1999;8:1939-1945.

57. Centers for Disease Control and Prevention. Progressive vaccinia in a military smallpox vaccinee—United States, 2009. MMWR Morb Mortal Wkly Rep. 2009;58:532-536.

58. Champness JN, Bennett MS, Wien F, et al. Exploring the active site of herpes simplex virus type-1 thymidine kinase by X-ray crystallography of complexes with aciclovir and other ligands. Proteins. 1998;32:350-361.

59. Chan DC, Fass D, Berger JM, Kim PS. Core structure of gp41 from the HIV envelope protein. Cell. 1997;89:263-273.

60. Chen C, Matthews TJ, McDanal CB, Bolognesi DP, Greenberg ML. A molecular clasp in the human immunodeficiency virus (HIV) type 1 TM protein determines the anti-HIV activity of gp41 derivatives: implications for viral fusion. J Virol. 1995;69:3771-3777.

61. Chen CH, Vasquez-Padua M, Cheng Y-C. Effect of anti-human immunodeficiency virus nucleoside analogs on mitochondrial DNA and its

implication for delayed toxicity. Mol Pharmacol. 1991;39:625-628.

62. Chen H, Beardsley GP, Coen DM. Mechanism of ganciclovir-induced chain termination revealed by resistant viral polymerase mutants with reduced exonuclease activity. Proc Natl Acad Sci U S A. 2014;111:17462-17467.

63. Chen H, Lawler JL, Filman DJ, Hogle JM, Coen DM. Resistance to a nucleoside analog antiviral drug from more rapid extension of drug-containing primers. MBio. 2021;12:e03492-20.

64. Chen X, Tsiang M, Yu F, et al. Modeling, analysis, and validation of a novel HIV integrase structure provide insights into the binding modes of potent integrase inhibitors. J Mol Biol. 2008;380:504-519.

65. Cheng G, Tian Y, Doehle B, et al. In vitro antiviral activity and resistance profile characterization of the hepatitis C virus NS5A inhibitor ledipasvir. Antimicrob Agents Chemother. 2016;60:1847-1853.

66. Cheng Y-C, Dutschman GE, Bastow KF, Sarngadharan MG, Ting RYC. Human immunodeficiency virus reverse transcriptase. General properties and its interactions with nucleoside triphosphate analogs. J Biol Chem. 1987;262:2187-2189.

67. Cherrington JM, Allen SJ, McKee BH, Chen MS. Kinetic analysis of the interaction between the diphosphate of (S)-1-3-hydroxy-2(phosphonylmethox)propyl)cytosine, ddCTP, AZTTP, and FIAUTP with human DNA polymerases β and γ. Biochem Pharmacol. 1994;48:1986-1988.

68. Chiou HC, Kumura K, Hu A, Kerns KM, Coen DM. Penciclovir-resistance mutations in the herpes simplex virus DNA polymerase gene. Antiviral Chem Chemother. 1995;6:281-288.

69. Cho A, Saunders OL, Butler T, et al. Synthesis and antiviral activity of a series of 1′-substituted 4-aza-7,9-dideazaadenosine C-nucleosides. Bioorg Med Chem Lett. 2012;22: 2705-2707.

70. Chou S. Rapid in vitro evolution of human cytomegalovirus UL56 mutations that confer letermovir resistance. Antimicrob Agents Chemother. 2015;59:6588-6593.

71. Chou S. A third component of the human cytomegalovirus terminase complex is involved in letermovir resistance. Antiviral Res. 2017;148:1-4.

72. Chou S. Comparison of cytomegalovirus terminase gene mutations selected after exposure to three distinct inhibitor compounds. Antimicrob Agents Chemother. 2017;61:e01325-17.

73. Chou S, Marousek GI. Accelerated evolution of maribavir resistance in a cytomegalovirus exonuclease domain II mutant. J Virol. 2008;82:246-253.

74. Chou S, Song K, Wu J, Bo T, Crumpacker C. Drug resistance mutations and associated phenotypes detected in clinical trials of maribavir for treatment of cytomegalovirus infection. J Infect Dis. 2022;226:576-584.

75. Choy KT, Wong AY, Kaewpreedee P, et al. Remdesivir, lopinavir, emetine, and homoharringtonine inhibit SARS-CoV-2 replication in vitro. Antiviral Res. 2020;178:104786.

76. Cihlar T, Votruba I, Horska K, Liboska R. Metabolism of 1-(S)-3-hydroxy-2-phosphonomethoxypropyl)cytosine (HPMPC) in human embryonic lung cells. Collect Czech Chem Commun. 1992;57:661-672.

77. Cleland A, Watson HG, Robertson P, Ludlam CA, Brown AJ. Evolution of zidovudine resistance-associated genotypes in human immunodeficiency virus type 1-infected patients. J Acquir Immune Defic Syndr Hum Retrovirol. 1996;12:6-18.

78. Coates JAV, Cammack N, Jenkinson HJ, et al. (-)-2′-deoxy-3′ thiacytidine is a potent, highly selective inhibitor of human immunodeficiency virus type 1 and type 2 replication in vitro. Antimicrob Agents Chemother. 1992;36:733-736.

79. Coates JAV, Cammack N, Jenkinson HJ, et al. The separated enantiomers of 2′-didexoxy-3′ thiacytidine (BCH-189) both inhibit human immunodeficiency virus replication in vitro. Antimicrob Agents Chemother. 1992;36:202-205.

80. Coburn CA, Meinke PT, Chang W, et al. Discovery of MK-8742: an HCV NS5A inhibitor with broad genotype activity. ChemMedChem. 2013;8:1930-1940.

81. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusson P. Identification of RANTES, MIP-1a, and MIP-1b as the major HIV-suppressive factors produced by CD8+ T cells. Science. 1995;270:1811-1815.

82. Coen DM. Acyclovir-resistant, pathogenic herpesviruses. Trends Microbiol. 1994;2: 481-485.

83. Coen DM, Schaffer PA. Two distinct loci confer resistance to acycloguanosine in herpes simplex virus type 1. Proc Natl Acad Sci U S A. 1980;77:2265-2269.

84. Coffin JM. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science. 1995;267:483-489.

85. Coffin JM. Population dynamics of HIV drug resistance. In: Richman DD, ed. Antiviral Drug Resistance. John Wiley & Sons; 1996:279-303.

86. Cohen KA, Hopkins J, Ingraham RH, et al. Characterization of the binding site for nevirapine (BI-RG-587), a nonnucleoside inhibitor of human immunodeficiency virus type-1 reverse transcriptase. J Biol Chem. 1991;266:14670-14674.

87. Collins JA, Thompson MG, Paintsil E, Ricketts M, Gedzior J, Alexander L. Competitive fitness of nevirapine-resistant human immunodeficiency virus type 1 mutants. J Virol. 2004;78:603-611.

88. Colonno R, Rose R, McLaren C, Thiry A, Parkin N, Friborg J. Identification of I50L as the signature atazanavir (ATV)-resistance mutation in treatment-naive HIV-1-infected patients receiving ATV-containing regimens. J Infect Dis. 2004;189:1802-1810.

89. Condra JH, Emini EA, Gotlib L, et al. Identification of the human immunodeficiency virus reverse transcriptase residues that contribute to the activity of diverse non-nucleoside inhibitors. Antimicrob Agents Chemother. 1992;36:1441-1446.

90. Corey L, Wald A, Patel R, et al. Once-daily valacyclovir to reduce the risk of transmission of genital herpes. N Engl J Med. 2004;350:11-20.

91. Cox RM, Wolf JD, Plemper RK. Therapeutically administered ribonucleoside analogue MK-4482/EIDD-2801 blocks SARS-CoV-2 transmission in ferrets. Nat Microbiol. 2021;6:11-18.

92. Craig JC, Grief C, Mills JS, Hockley D, Duncan IB, Roberts NA. Effects of a specific inhibitor of HIV proteinase (Ro 31-8959) on virus maturation in a chronically infected promonocytic cell line (U1). Antiviral Chem Chemother. 1991;2:181-186.

93. Dangerfield TL, Huang NZ, Johnson KA. Remdesivir is effective in combating COVID-19 because it is a better substrate than ATP for the viral RNA-dependent RNA polymerase. iScience. 2020;23:101849.

94. Davies WL, Grunert RR, Haff RF, et al. Antiviral activity of 1-adamantanamine (amantadine). Science. 1964;144:862-863.

95. de Jong MD, Tran TT, Truong HK, et al. Oseltamivir resistance during treatment of influenza A (H5N1) infection. N Engl J Med. 2005;353:2667-2672.

96. De Meyer S, Ghys A, Foster GR, et al. Analysis of genotype 2 and 3 hepatitis C virus variants in patients treated with telaprevir demonstrates a consistent resistance profile across genotypes. J Viral Hepat. 2013;20:395-403.

97. de Ronde A, van Dooren M, van Der Hoek L, et al. Establishment of new transmissible and drug-sensitive human immunodeficiency virus type 1 wild types due to transmission of nucleoside analogue-resistant virus. J Virol. 2001;75:595-602.

98. Dharan NJ, Gubareva LV, Meyer JJ, et al. Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. JAMA. 2009;301:1034-1041.

99. Di Bisceglie AM, Conjeevaram HS, Fried MW, et al. Ribavirin as therapy for chronic hepatitis C. A randomized, double-blind, placebo-controlled trial. Ann Intern Med. 1995;123:897-903.

100. Diallo K, Götte M, Wainberg MA. Molecular impact of the M184V mutation in human immunodeficiency virus type 1 reverse transcriptase. Antimicrob Agents Chemother. 2003;47:337-3383.

101. Dodson GW, Wlodawer A. Catalytic triads and their relatives. Trends Biochem Sci. 1988;29:347-352.

102. Dorr P, Westby M, Dobbs S, et al. Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. Antimicrob Agents Chemother. 2005;49:4721-4732.

103. Dou D, Revol R, Ostbye H, Wang H, Daniels R. Influenza A virus cell entry, replication, virion assembly and movement. Front Immunol. 2018;9:1581.

104. Douglas CM, Barnard R, Holder D, et al. Letermovir resistance analysis in a clinical trial of cytomegalovirus prophylaxis for hematopoietic stem cell transplant recipients. J Infect Dis. 2020;221:1117-1126.

105. Doyon L, Croteau G, Thibeault D, Poulin F, Pilote L, Lamarre D. Second locus involved in human immunodeficiency virus type 1 resistance to protease inhibitors. J Virol. 1996;70: 3763-3769.

106. Drake JW, Holland JJ. Mutation rates among RNA viruses. Proc Natl Acad Sci U S A. 1999;96:13910-13913.

107. Dubay J, Roberts S, Brody B, Hunter E. Mutations in the leucine zipper of HIV-1 transmembrane glycoprotein affect fusion and infectivity. J Virol. 1992;66:4748-4756.

108. Duff KC, Ashley RH. The transmembrane domain of influenza A M2 protein forms amantadine-sensitive proton channels in planar lipid bilayers. Virology. 1992;190:485-489.

109. Duraffour S, Andrei G, Topalis D, et al. Mutations conferring resistance to viral DNA polymerase inhibitors in camelpox virus give different drug-susceptibility profiles in vaccinia virus. J Virol. 2012;86:7310-7325.

110. Duraffour S, Lorenzo MM, Zoller G, et al. ST-246 is a key antiviral to inhibit the viral F13L phospholipase, one of the essential proteins for orthopoxvirus wrapping. J Antimicrob Chemother. 2015;70:1367-1380.

111. Earnshaw DL, Bacon TH, Darlison SJ, Edmonds K, Perkins RM, Vere Hodge RA. Mode of antiviral action of penciclovir in MRC-5 cells infected with herpes simplex virus type 1 (HSV-1), HSV-2, and varicella-zoster virus. Antimicrob Agents Chemother. 1992;36:2747-2757.

112. Eastman RT, Roth JS, Brimacombe KR, et al. Remdesivir: a review of its discovery and development leading to emergency use authorization for treatment of COVID-19. ACS Cent Sci. 2020;6:672-683.

113. Eckerle LD, Lu X, Sperry SM, Choi L, Denison MR. High fidelity of murine hepatitis replication is decreased in nsp14 exoribonuclease mutants. J Virol. 2007;81: 12135-12144.

114. Elion GB, Furman PA, Fyfe JA, de Miranda P, Beauchamp L, Schaeffer HJ. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. Proc Natl Acad Sci U S A. 1977;74:5716-5720.

115. Emu B, Fessel J, Schrader S, et al. Phase 3 study of ibalizumab for multidrug-resistant HIV-1. N Engl J Med. 2018;379:645-654.

116. Engleke M, Mills K, Seitz S, et al. Characterization of a hepatitis B and hepatitis D virus receptor binding site. Hepatology. 2006;43:750-760.

117. Erice A, Mayers DL, Strike DG, Balfour HJ. Primary infection with zidovudine-resistant human immunodeficiency virus type 1. N Engl J Med. 1993;328:1163-1165.

118. Erickson JW, Meidhart DJ, Van Drie J, et al. Design, activity, and a 2.8 Å crystal structure of a C2 symmetric inhibitor complexed to HIV-1 protease. Science. 1990;249:527-533.

119. Eriksson B, Öberg B, Wahren B. Pyrophosphate analogs as inhibitors of DNA polymerases of cytomegalovirus, herpes simplex virus and cellular origin. Biochim Biophys Acta. 1982;696:115-123.

120. Erlich KS, Mills J, Chatis P, et al. Acyclovir-resistant herpes simplex virus infections in patients with the acquired immunodeficiency syndrome. N Engl J Med. 1989;320:293-296.

121. European AIDS Clinical Society. Guidelines Version 11.1. October, 2022.

122. European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol. 2017;67:370-398.

123. European Medicines Agency. Assessment report: Hepcludex. 2020.

124. Faletto MB, Miller WH, Garvey EP, St Clair MH, Daluge SM, Good SS. Unique intracellular activation of the potent anti-human immunodeficiency virus agent 1592U89. Antimicrob Agents Chemother. 1997;41:1099-1107.

125. Feld JJ, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature. 2005;436:967-972.

126. Feld JJ, Jacobson IM, Hezode C, et al. Sofosbuvir and velpatasvir for HCV genotype 1, 2, 4, 5, and 6 infection. N Engl J Med. 2015;373:2599-2607.

127. Feng M, Sachs NA, Xu M, et al. Doravirine suppresses common nonnucleoside reverse transcriptase inhibitor-associated mutants at clinically relevant concentrations. Antimicrob Agents Chemother. 2016;60:2241-2247.

128. Ferenci P, Bernstein D, Lalezari J, et al. ABT-450/r-ombitasvir and dasabuvir with or without ribavirin for HCV. N Engl J Med. 2014;370:1983-1992.

129. Focosi D, Maggi F, McConnell S, Casadevall A. Very low levels of remdesivir resistance in SARS-COV-2 genomes after 18 months of massive usage during the COVID19 pandemic: A GISAID exploratory analysis. Antiviral Res. 2022;198:105247.

130. Forns X, Lee SS, Valdes J, et al. Glecaprevir plus pibrentasvir for chronic hepatitis C virus genotype 1, 2, 4, 5, or 6 infection in adults with compensated cirrhosis (EXPEDITION-1): a single-arm, open-label, multicentre phase 3 trial. Lancet Infect Dis. 2017;17:1062-1068.

131. Foster GR, Afdhal N, Roberts SK, et al. Sofosbuvir and velpatasvir for HCV genotype 2 and 3 infection. N Engl J Med. 2015;373:2608-2617.

132. Foster GR, Agarwal K, Cramp ME, et al. Elbasvir/grazoprevir and sofosbuvir for hepatitis C virus genotype 3 infection with compensated cirrhosis: a randomized trial. Hepatology. 2018;67:2113-2126.

133. Foster SA, Parker S, Lanier R. The role of brincidofovir in preparation for a potential smallpox outbreak. Viruses. 2017;9:320-327.

134. Franklin MC, Wang J, Steitz TA. Structure of the replicating complex of a pol α family DNA polymerase. Cell. 2001;105:657-667.

135. Freed EO, Martin MA. HIVs and their replication. In: Knipe DM, Howley PM, Griffin DE, et al., eds. Fields Virology. 5th ed. Wolters Kluwer/Lippincott Williams & Wilkins; 2007.

136. Fridell RA, Wang C, Sun JH, et al. Genotypic and phenotypic analysis of variants resistant to hepatitis C virus nonstructural protein 5A replication complex inhibitor BMS-790052 in humans: in vitro and in vivo correlations. Hepatology. 2011;54:1924-1935.

137. Fridman A, Murgolo NJ, Liaw A, et al. Molnupiravir increases random SARS-CoV-2 RNA errors, without selection for resistance. Conference on Retroviruses and Opportunistic Infections. 2022.

138. Fu L, Ye F, Feng Y, et al. Both boceprevir and GC376 efficaciously inhibit SARS-CoV-2 by targeting its main protease. Nat Commun. 2020;11:4417.

139. Fujisaki S, Yokomaku Y, Shiino T, et al. Outbreak of infections by hepatitis B virus genotype A and transmission of genetic drug resistance in patients coinfected with HIV-1 in Japan. J Clin Microbiol. 2011;49:1017-1024.

140. Furman PA, Fyfe JA, St Clair MH, et al. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc Natl Acad Sci U S A. 1986;83:8333-8337.

141. Furman, PA Sofia MJ. *The Discovery of Sofosbuvir: A Liver-Targeted Nucleotide Prodrug for the Treatment and Cure of HCV. Vol 1*. Springer; 2019.

142. Fyfe JA, Keller PM, Furman PA, Miller RL, Elion GB. Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound 9-(2-hydroxyethoxymethyl)guanine. J Biol Chem. 1978;253:8721-9727.

143. Gandhi S, Klein J, Robertson A, et al. De novo emergence of a remdesivir resistance mutation during treatment of persistent SARS-CoV-2 infection in an immunocompromised patient: a case report. Nat Commun. 2022;13:1547.

144. Gao M, Nettles RE, Belema M, et al. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. Nature. 2010;465:96-100.

145. Gao Q, Gu Z-X, Parniak MA, Li X-G, Wainberg MA. *In vitro* selection of variants of human immunodeficiency virus type 1 resistant to 3′-azido-3′-deoxythymidine and 2′,-3′-dideoxyinosine. J Virol. 1992;66:12-19.

146. Garcia-Perez J, Rueda P, Staropoli I, et al. New insights into the mechanisms whereby low molecular weight CCR5 ligands inhibit HIV-1 infection. J Biol Chem. 2011;286:4978-4990.

147. Ge D, Fellay J, Thompson AJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009;461:399-401.

148. Geddawy A, Ibrahim YF, Elbahie NM, Ibrahim MA. Direct acting anti-hepatitis C virus drugs: clinical pharmacology and future direction. J Transl Int Med. 2017;5:8-17.

149. Gelderblom H. Assembly and morphology of HIV: potential effects of structure on viral function. AIDS. 1991;5:617-638.

150. Gentry BG, Kamil JP, Coen DM, Zemlicka J, Drach JC. Stereoselective phosphorylation of cyclopropavir by pUL97 and competitive inhibition by maribavir. Antimicrob Agents Chemother. 2010;54:3093-3098.

151. Germershausen J, Bostedor R, Field AK, et al. A comparison of the antiviral agents 2′-nor-2′-deoxyguanosine and acyclovir: uptake and phosphorylation in tissue culture and kinetics of *in vitro* inhibition of viral and cellular DNA polymerases by their respective triphosphates. Biochem Biophys Res Commun. 1983;116:360-367.

152. Gibson SJ, Lindh JM, Riter TR, et al. Plasmacytoid dendritic cells produce cytokines and mature in response to the TLR7 agonists, imiquimod and resiquimod. Cell Immunol. 2002;218:74-86.

153. Gilbert C, Bestman-Smith J, Boivin G. Resistance of herpesviruses to antiviral drugs: clinical impacts and molecular mechanisms. Drug Resist Updat. 2002;5:88-114.

154. Goldner T, Hewlett G, Ettischer N, Ruebsamen-Schaeff H, Zimmermann H, Lischka P. The novel anticytomegalovirus compound AIC246 (Letermovir) inhibits human cytomegalovirus replication through a specific antiviral mechanism that involves the viral terminase. J Virol. 2011;85:10884-10893.

155. Gordon CJ, Tchesnokov EP, Schinazi RF, Gotte M. Molnupiravir promotes SARS-CoV-2 mutagenesis via the RNA template. J Biol Chem. 2021;297:100770.

156. Gordon CJ, Tchesnokov EP, Woolner E, et al. Remdesivir is a direct-acting antiviral that inhibits RNA-dependent RNA polymerase from severe acute respiratory syndrome coronavirus 2 with high potency. J Biol Chem. 2020;295:6785-6797.

157. Gottlieb RL, Vaca CE, Paredes R, et al. Early remdesivir to prevent progression to severe Covid-19 in outpatients. N Engl J Med. 2022;386:305-315.

158. Gottwein JM, Scheel TK, Jensen TB, Ghanem L, Bukh J. Differential efficacy of protease inhibitors against HCV genotypes 2a, 3a, 5a, and 6a NS3/4A protease recombinant viruses. Gastroenterology. 2011;141:1067-1079.

159. Graepel KW, Lu X, Case JB, Sexton NR, Smith EC, Denison MR. Proofreading-deficient coronaviruses adapt for increased fitness over long-term passage without reversion of exoribonuclease-inactivating mutations.MBio. 2017;8:e01503-16.

160. Griffiths A, Coen DM. High-frequency phenotypic reversion and pathogenicity of an acyclovir-resistant herpes simplex virus mutant. J Virol. 2003;77:2282-2286.

161. Gripon P, Cannie I, Urban S. Efficient inhibition of hepatitis B virus infection by acylated peptides derived from the large viral surface protein. J Virol. 2005;79:1613-1622.

162. Grosenbach DW, Honeychurch K, Rose EA, et al. Oral tecovirimat for the treatment of smallpox. N Engl J Med. 2018;379:44-53.

163. Group RC. Dexamethasone in hospitalized patients with Covid-19. N Engl J Med. 2021;384:693-704.

164. Gubareva LV, Bethell RC, Hart GJ, Murti KG, Penn CR, Webster RG. Characterization of mutants of influenza A virus selected with the neuraminidase inhibitor 4-guanidino-Neu5Ac2en. J Virol. 1996;70:1818-1827.

165. Gubareva LV, Webster RG, Hayden FG. Comparison of the activities of zanamivir, oseltamivir, and RWJ-270201 against clinical isolates of influenza virus and neuraminidase inhibitor-resistant variants. Antimicrob Agents Chemother. 2001;45:3403-3408.

166. Guo Q, Ho HT, Dicker I, et al. Biochemical and genetic characterizations of a novel human immunodeficiency virus type 1 inhibitor that blocks gp120-CD4 interactions. J Virol. 2003;77:10528-10536.

167. Guoqiang Wang JM, Jiang L-J, Gai Y, Long J, Bin Wang KFM, Or YS. Discovery and Development of the Next-Generation HCV NS3 Protease Inhibitor Glecaprevir. Vol 31. Topics in Medicinal Chemistry. Springer; 2019.

168. Hamirally S, Kamil JP, Ndassa-Colday YM, et al. Viral mimicry of Cdc2/cyclin-dependent kinase 1 mediates disruption of nuclear lamina during human cytomegalovirus nuclear egress. PLoS Pathog. 2009;5:e1000275.

169. Han B, Parhy B, Lu J, et al. In vitro susceptibility of hepatitis C virus genotype 1 through 6 clinical isolates to the pangenotypic NS3/4A

inhibitor voxilaprevir. J Clin Microbiol. 2019;57:e01844-18.

170. Harper S, McCauley JA, Rudd MT, et al. Discovery of MK-5172, a macrocyclic hepatitis C virus NS3/4a protease inhibitor. ACS Med Chem Lett. 2012;3:332-336.

171. Hart GJ, Orr DC, Penn CR, et al. Effects of (-)-2′-deoxy-3′-thiacytidine (3TC) 5′ triphosphate on human immunodeficiency virus reverse transcriptase and mammalian DNA polymerases alpha, beta, and gamma. Antimicrob Agents Chemother. 1992;36:1688-1694.

172. Hay AJ. Amantadine and rimantadine—mechanisms. In: Richman DD, ed. Antiviral Drug Resistance. John Wiley & Sons; 1996:43-58.

173. Hay AJ, Wolstenholme AJ, Skehel JJ, Smith MH. The molecular basis of the specific anti-influenza action of amantadine. EMBO J. 1985;4:3021-3024.

174. Hayden FG. Amantadine and rimantadine resistance in influenza A viruses. Curr Opin Infect Dis. 1994;7:674-677.

175. Hayden FG, Atmar RL, Schilling M, et al. Use of the selective oral neuraminidase inhibitor oseltamivir to prevent influenza. N Engl J Med. 1999;341:1336-1343.

176. Hayden FG, de Jong MD. Emerging influenza antiviral resistance threats. J Infect Dis. 2011;203:6-10.

177. Hayden FG, Gubareva LV, Monto AS, et al. Inhaled zanamivir for the prevention of influenza in families. Zanamivir Family Study Group. N Engl J Med. 2000;343:1282-1289.

178. Hayden FG, Osterhaus AD, Treanor JJ, et al. Efficacy and safety of the neuraminidase inhibitor zanamivir in the treatment of influenzavirus infections. GG167 Influenza Study Group. N Engl J Med. 1997;337:874-880.

179. Hayden FG, Sugaya N, Hirotsu N, et al. Baloxavir marboxil for uncomplicated influenza in adults and adolescents. N Engl J Med. 2018;379:913-923.

180. Hazuda DJ, Felock P, Witmer M, et al. Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. Science. 2000;287:646-650.

181. He Z, He Y-S, Kim Y, et al. The human cytomegalovirus UL97 protein is a protein kinase that autophosphorylates on serines and threonines. J Virol. 1997;71:405-411.

182. Heming JD, Conway JF, Homa FL. Herpesvirus capsid assembly and DNA packaging. Adv Anat Embryol Cell Biol. 2017;223:119-142.

183. Hemmi H, Kaisho T, Takeuchi O, et al. Small anti-viral compounds activate immune cells *via* the TLR7 MyD88-dependent signaling pathway. Nat Immunol. 2002;3: 196-200.

184. Herlocher ML, Truscon R, Elias S, et al. Influenza viruses resistant to the antiviral drug oseltamivir: transmission studies in ferrets. J Infect Dis. 2004;190:1627-1630.

185. Herrmann EC Jr, Herrmann JA. A working hypothesis—virus resistance development as an indicator of specific antiviral activity. Ann N Y Acad Sci. 1977;284:632-637.

186. Herschhorn A, Gu C, Moraca F, et al. The beta20-beta21 of gp120 is a regulatory switch for HIV-1 Env conformational transitions. Nat Commun. 2017;8:1049.

187. Hill JA. Human herpesvirus 6 in transplant recipients: an update on diagnostic and treatment strategies. Curr Opin Infect Dis. 2019;32:584-590.

188. Ho H-T, Woods KL, Bronson JJ, De Boeck H, Martin JC, Hitchcock MJM. Intracellular metabolism of the antiherpes agent (*S*)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine. Mol Pharmacol. 1992;41:197-202.

189. Holsinger LJ, Lamb RA. Influenza virus M2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. Virology. 1991;183:32-43.

190. Hostetler KY. Alkoxyalkyl prodrugs of acyclic nucleoside phosphonates enhance oral antiviral activity and reduce toxicity: current state of the art. Antiviral Res. 2009;82:A84-A98.

191. Howe AY, Venkatraman S. The discovery and development of boceprevir: a novel, first-generation inhibitor of the hepatitis C virus NS3/4A serine protease. J Clin Transl Hepatol. 2013;1:22-32.

192. Huang L, Hwang J, Sharma SD, et al. Hepatitis C virus nonstructural protein 5A (NS5A) is an RNA-binding protein. J Biol Chem. 2005;280:36417-36428.

193. Huang L, Ishii KK, Zuccola H, et al. The enzymological basis for resistance of herpesvirus DNA polymerase mutants to acyclovir: relationship to the structure of α-like DNA polymerases. Proc Natl Acad Sci U S A. 1999;96:447-452.

194. Hume AJ, Finkel JS, Kamil JP, Coen DM, Culbertson MR, Kalejta RF. Phosphorylation of retinoblastoma protein by viral protein with cyclin-dependent kinase function. Science. 2008;320:797-799.

195. Humeniuk R, Mathias A, Kirby BJ, et al. Pharmacokinetic, pharmacodynamic, and drug-interaction profile of remdesivir, a SARS-CoV-2 replication inhibitor. Clin Pharmacokinet. 2021;60:569-583.

196. Hutson CL, Kondas AV, Mauldin MR, et al. Pharmacokinetics and efficacy of a potential smallpox therapeutic, brincidofovir, in a lethal monkeypox virus animal model. mSphere. 2021;6:e00927-20.

197. Hwang CB, Chen HJ. An altered spectrum of herpes simplex virus mutations mediated by an antimutator DNA polymerase. Gene. 1995;152:191-193.

198. Hwang CBC, Horsburgh B, Pelosi E, Roberts S, Digard P, Coen DM. A net +1 frameshift permits synthesis of thymidine kinase from a drug-resistant herpes simplex virus mutant. Proc Natl Acad Sci U S A. 1994;91:5461-5465.

199. Hwang YT, Liu B-Y, Coen DM, Hwang CBC. Effects of mutations in the Exo III motif of the herpes simplex virus DNA polymerase gene on enzyme activities, viral replication, and replication fidelity. J Virol. 1997;71:7791-7798.

200. Iglesias-Ussel M, Casado C, Yuste E, Olivares I, López-Galindez C. *In vitro* analysis of human immunodeficiency virus type 1 resistance to nevirapine and fitness determination of resistant variants. J Gen Virol. 2002;83:93-101.

201. Ikematsu H, Hayden FG, Kawaguchi K, et al. Baloxavir marboxil for prophylaxis against influenza in household contacts. N Engl J Med. 2020;383:309-320.

202. Ilsley DD, Lee S-H, Miller WH, Kuchta RD. Acyclic guanosine analogues inhibit DNA polymerases α, δ, and ε with very different potencies and have unique mechanisms of action. Biochemistry. 1995;34:2504-2510.

203. Ives JA, Carr JA, Mendel DB, et al. The H274Y mutation in the influenza A/H1N1 neuraminidase active site following oseltamivir phosphate treatment leaves virus severely compromised both in vitro and in vivo. Antiviral Res. 2002;55:307-317.

204. Jacobson IM, Lawitz E, Gane EJ, et al. Efficacy of 8 weeks of sofosbuvir, velpatasvir, and voxilaprevir in patients with chronic HCV infection: 2 phase 3 randomized trials. Gastroenterology. 2017;153:113-122.

205. Jayk Bernal A, Gomes da Silva MM, Musungaie DB, et al. Molnupiravir for oral treatment of covid-19 in nonhospitalized patients. N Engl J Med. 2022;386:509-520.

206. Jing X, Ma C, Ohigashi Y, et al. Functional studies indicate amantadine binds to the pore of the influenza A virus M2 proton-selective ion channel. Proc Natl Acad Sci U S A. 2008;105:10967-10972.

207. Johnson VA, Brun-Vézinet F, Clotet B, et al. Update of the drug resistance mutations in HIV-1: December 2010. Top HIV Med. 2010;18:156-163.

208. Jurk M, Heil F, Vollmer J, et al. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat Immunol. 2002;3:499.

209. Kabinger F, Stiller C, Schmitzova J, et al. Mechanism of molnupiravir-induced SARS-CoV-2 mutagenesis. Nat Struct Mol Biol. 2021;28:740-746.

210. Kalil AC, Patterson TF, Mehta AK, et al. Baricitinib plus remdesivir for hospitalized adults with Covid-19. N Engl J Med. 2021;384:795-807.

211. Kamil JP, Hume AJ, Jurak I, Münger K, Kalejta RF, Coen DM. Human papillomavirus 16 E7 inactivator of retinoblastoma family proteins complements human cytomegalovirus lacking UL97 protein kinase. Proc Natl Acad Sci U S A. 2009;106:16823-16828.

212. Kellam P, Boucher CA, Larder BA. Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine. Proc Natl Acad Sci U S A. 1992;89:1934-1938.

213. Kempf DJ, Marsh KC, Denissen JF, et al. ABT-538 is a potent inhibitor of human immunodeficiency virus protease and has high oral bioavailability in humans. Proc Natl Acad Sci U S A. 1995;92:2484-2488.

214. Kempf DJ, Marsh KC, Paul DA, et al. Antiviral and pharmacokinetic properties of C2 symmetric inhibitors of the human immunodeficiency virus type 1 protease. Antimicrob Agents Chemother. 1991;35:2209-2214.

215. Kempf DJ, Norbeck DW, Codacovi L, et al. Structure-based, *C*2 symmetric inhibitors of HIV protease. J Med Chem. 1990;33:2687-2689.

216. Kieffer TL, Sarrazin C, Miller JS, et al. Telaprevir and pegylated interferon-alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. Hepatology. 2007;46:631-639.

217. Kim CU, Lew W, Williams MA, et al. Structure-activity relationship studies of novel carbocyclic influenza neuraminidase inhibitors. J Med Chem. 1998;41:2451-2460.

218. Kirschberg TA, Squires NH, Yang H, et al. Novel, sulfonamide linked inhibitors of the hepatitis C virus NS3 protease. Bioorg Med Chem Lett. 2014;24:969-972.

219. Kleymann G, Fischer R, Betz UAK, et al. New helicase-primase inhibitors as drug candidates for the treatment of herpes simplex disease. Nat Med. 2002;8:392-398.

220. Kohlstaedt LA, Wang J, Friedman JM, Rice PA, Steitz TA. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science. 1992;256:1783-1790.

221. Kohno S, Yen MY, Cheong HJ, et al. Phase III randomized, double-blind study comparing single-dose intravenous peramivir with oral oseltamivir in patients with seasonal influenza virus infection. Antimicrob Agents Chemother. 2011;55:5267-5276.

222. Kokic G, Hillen HS, Tegunov D, et al. Mechanism of SARS-CoV-2 polymerase stalling by remdesivir. Nat Commun. 2021;12:279.

223. Komazin G, Ptak RG, Emmer BT, Townsend LB, Drach JC. Resistance of human cytomegalovirus to the benzimidazole L-ribonucleoside maribavir maps to UL27. J Virol. 2003;77:11499-11506.

224. Kondru R, Zhang J, Ji C, et al. Molecular interactions of CCR5 with major classes of small-molecule anti-HIV CCR5 antagonists. Mol Pharmacol. 2008;73:789-800.

225. Koszalka GW, Johnson NW, Good SS, et al. Preclinical and toxicology studies of 1263W94, a potent and selective inhibitor of human cytomegalovirus replication. Antimicrob Agents Chemother. 2002;46:2373-2380.

226. Kozal M, Aberg J, Pialoux G, et al. Fostemsavir in adults with multidrug-resistant HIV-1 infection. N Engl J Med. 2020;382:1232-1243.

227. Krischuns T, Lukarska M, Naffakh N, Cusack S. Influenza virus RNA-dependent RNA polymerase and the host transcriptional apparatus. Annu Rev Biochem. 2021;90:321-348.

228. Krishnan P, Beyer J, Mistry N, et al. In vitro and in vivo antiviral activity and resistance profile of ombitasvir, an inhibitor of hepatitis C virus NS5A. Antimicrob Agents Chemother. 2015;59:979-987.

229. Krosky PM, Baek MC, Coen DM. The human cytomegalovirus UL97 protein kinase, an antiviral drug target, is required at the stage of nuclear egress. J Virol. 2003;77:905-914.

230. Kumar D, Ison MG, Mira JP, et al. Combining baloxavir marboxil with standard-of-care neuraminidase inhibitor in patients hospitalised with severe influenza (FLAGSTONE): a randomised, parallel-group, double-blind, placebo-controlled, superiority trial. Lancet Infect Dis. 2022;22:718-730.

231. Kuny CV, Kalejta RF. Human cytomegalovirus can procure deoxyribonucleotides for viral DNA replication in the absence of retinoblastoma protein phosphorylation. J Virol 2016;90:8634-8643.

232. Kuritzkes DR, Quinn JB, Benoit SL, et al. Drug resistance and virologic response in NUCA 3001, a randomized trial of lamivudine (3TC) versus zidovudine (ZDV) versus ZDV plus 3TC in previously untreated patients. AIDS. 1996;10:975-981.

233. Kwo PY, Poordad F, Asatryan A, et al. Glecaprevir and pibrentasvir yield high response rates in patients with HCV genotype 1-6 without cirrhosis. J Hepatol. 2017;67:263-271.

234. Lam AM, Espiritu C, Bansal S, et al. Genotype and subtype profiling of PSI-7977 as a nucleotide inhibitor of hepatitis C virus. Antimicrob Agents Chemother. 2012;56: 3359-3368.

235. Lam AM, Murakami E, Espiritu C, et al. PSI-7851, a pronucleotide of beta-D-2′-deoxy-2′-fluoro-2′′-C-methyluridine monophosphate, is a potent and pan-genotype inhibitor of hepatitis C virus replication. Antimicrob Agents Chemother. 2010;54:3187-3196.

236. Lamarre D, Anderson PC, Bailey M, et al. An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. Nature. 2003;426:186-189.

237. Landro JA, Raybuck SA, Luong YP, et al. Mechanistic role of an NS4A peptide cofactor with the truncated NS3 protease of hepatitis C virus: elucidation of the NS4A stimulatory effect via kinetic analysis and inhibitor mapping. Biochemistry. 1997;36:9340-9348.

238. Larder BA, Coates KE, Kemp SD. Zidovudine-resistant human immunodeficiency virus selected by passage in cell culture. J Virol. 1991;65:5232-5236.

239. Larder BA, Darby G. Virus drug-resistance: mechanisms and consequences. Antiviral Res. 1984;4:1-42.

240. Larder BA, Darby G, Richman DD. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science. 1989;243:1731-1734.

241. Larder BA, Kellam P, Kemp SD. Zidovudine resistance predicted by direct detection of mutations in DNA from HIV-infected lymphocytes. AIDS. 1991;5:137-144.

242. Larder BA, Kemp SD. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science. 1989;246:1155-1158.

243. Laver WG, Bischofberger N, Webster RG. Disarming flu viruses. Sci Am. 1999;280:78-87.

244. Lawitz E, Mangia A, Wyles D, et al. Sofosbuvir for previously untreated chronic hepatitis C infection. N Engl J Med. 2013;368:1878-1887.

245. Lawitz E, Sulkowski MS, Ghalib R, Rodriguez-Torres M, et al. Simeprevir plus sofosbuvir, with or without ribavirin, to treat chronic infection with hepatitis C virus genotype 1 in non-responders to pegylated interferon and ribavirin and treatment-naive patients: the COSMOS randomised study. Lancet. 2014;384:1756-1765.

246. Lawitz EJ, Dvory-Sobol H, Doehle BP, et al. Clinical resistance to velpatasvir (GS-5816), a novel pan-genotypic inhibitor of the hepatitis C virus NS5A protein. Antimicrob Agents Chemother. 2016;60:5368-5378.

247. Lawless MK, Barney S, Guthrie K, Bucy TB, Petteway SR Jr, Merutka G. HIV-1 membrane fusion mechanism: structural studies of the interactions between biologically active peptides from gp41. Biochemistry. 1996;35:13697-13708.

248. Lederman MM, Penn-Nicholson A, Cho M, Mosier D. Biology of CCR5 and its role in HIV infection and treatment. JAMA. 2006;296:815-826.

249. Lee H, Hanes J, Johnson KA. Toxicity of nucleoside analogues used to treat AIDS and the selectivity of the mitochondrial DNA polymerase. Biochemistry. 2003;23:14711-14719.

250. Leidner F, Yilmaz NK, Schiffer CA. Deciphering complex mechanisms of resistance and loss of potency through coupled molecular dynamics and machine learning. J Chem Theory Comput. 2021;17(4):2054-2064.

251. Lemm JA, O'Boyle D II, Liu M, et al. Identification of hepatitis C virus NS5A inhibitors. J Virol. 2010;84:482-491.

252. Lennerstrand J, Hertogs K, Stammers DK, Larder BA. Correlation between viral resistance to zidovudine and resistance at the reverse transcriptase level for a panel of human immunodeficiency virus type 1 mutants. J Virol. 2001;75:7202-7205.

253. Lennox JL, Landovitz RJ, Ribaudo HJ, et al. Efficacy and tolerability of 3 nonnucleoside reverse transcriptase inhibitor-sparing antiretroviral regimens for treatment-naive volunteers infected with HIV-1: a randomized, controlled equivalence trial. Ann Intern Med. 2014;161:461-471.

254. LeSeyec J, Chouteau P, Cannie I, Guguen-Guillouzo C, Gripon P. Infection process of the hepatitis B virus depends on the presence of a defined sequence in the pre-S1 domain. J Virol. 1999;81:5573-5578.

255. Li JX, Coen DM. Pharmacology of viral infections. In: Golan DE, Armstrong EJ, Armstrong AW, eds. Principles of Pharmacology: The pathophysiologic basis of drug therapy. 4th ed. Wolters Kluwer Health; 2017:694-721.

256. Li K, Foy E, Ferreon JC, et al. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc Natl Acad Sci U S A. 2005;102:2992-2997.

257. Li W-X, Escarpe PA, Eisenberg EJ, et al. Identification of GS4104 as an orally bioavailable prodrug of the influenza neuraminidase inhibitor GS4701. Antimicrob Agents Chemother. 1998;42:647-653.

258. Li Z, Zhou N, Sun Y, et al. Activity of the HIV-1 attachment inhibitor BMS-626529, the active component of the prodrug BMS-663068, against CD4-independent viruses and HIV-1 envelopes resistant to other entry inhibitors. Antimicrob Agents Chemother. 2013;57:4172-4180.

259. Lin K, Perni RB, Kwong AD, Lin C. VX-950, a novel hepatitis C virus (HCV) NS3-4A protease inhibitor, exhibits potent antiviral activities in HCV replicon cells. Antimicrob Agents Chemother. 2006;50:1813-1822.

260. Lin P-F, Blair W, Wang T, et al. A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. Proc Natl Acad Sci U S A. 2003;100:11013-11018.

261. Link JO, Rhee MS, Tse WC, et al. Clinical targeting of HIV capsid protein with a long-acting small molecule. Nature. 2020;584:614-618.

262. Lischka P, Hewlett G, Wunberg T, et al. In vitro and in vivo activities of the novel anticytomegalovirus compound AIC246. Antimicrob Agents Chemother. 2010;54:1290-1297.

263. Little SJ. Transmission and prevalence of HIV resistance among treatment-naive subjects. Antivir Ther. 2000;5:33-40.

264. Little SJ, Frost SD, Wong JK, et al. Persistence of transmitted drug resistance among subjects with primary human immunodeficiency virus infection. J Virol. 2008;82:5510-5518.

265. Littler E, Stuart AD, Chee MS. Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. Nature. 1992;358:160-162.

266. Liu C, Eichelberger MD, Compans RW, Air GM. Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. J Virol. 1995;69:1099-1106.

267. Liu R, Curry S, McMonagle P, et al. Susceptibilities of genotype 1a, 1b, and 3 hepatitis C virus variants to the NS5A inhibitor elbasvir. Antimicrob Agents Chemother. 2015;59:6922-6929.

268. Liu R, Paxton WA, Choe S, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell. 1996;86:367-378.

269. Liu S, Knafels JD, Chang JS, et al. Crystal structure of the herpes simplex virus 1 DNA polymerase. J Biol Chem. 2006;281:18193-18200.

270. Locarnini S, Bowden S. Drug resistance in antiviral therapy. Clin Liver Dis. 2010;14:439-459.

271. Lu J, Sista P, Giguel F, Greenberg M, Kuritzkes DR. Relative replicative fitness of human immunodeficiency virus type 1 mutants resistant to enfuvirtide (T-20). J Virol. 2004;78:4628-4637.

272. Lurain NS, Spafford LE, Thompson KD. Mutation in the UL97 open reading frame of human cytomegalovirus strains resistant to ganciclovir. J Virol. 1994;68:4427-4431.

273. Lye MF, Wilkie AR, Coen DM. Getting to and through the inner nuclear membrane during herpesvirus nuclear egress. Curr Opin Cell Biol. 2017;46:9-16.

274. Ma C, Sacco MD, Hurst B, et al. Boceprevir, GC-376, and calpain inhibitors II, XII inhibit SARS-CoV-2 viral replication by targeting the viral main protease. Cell Res. 2020;30:678-692.

275. Ma H, Jiang WR, Robledo N, et al. Characterization of the metabolic activation of hepatitis C virus nucleoside inhibitor beta-D-2′-Deoxy-2′-fluoro-2′-C-methylcytidine (PSI-6130) and identification of a novel active 5'-triphosphate species. J Biol Chem. 2007;282:29812-29820.

276. Ma JD, Lee KC, Kuo GM. HLA-B*5701 testing to predict abacavir hypersensitivity. PLoS Curr. 2010;7:RRN1023.

277. Maekawa S, Enomoto N. Viral factors influencing the response to the combination therapy of peginterferon plus ribavirin in chronic hepatitis C. J Gastroenterol. 2009;44: 1009-1015.

278. Mai Le Q, Kiso M, Someya K, et al. Isolation of drug-resistant H5N1 virus. Nature. 2005;437:1108.

279. Malone B, Campbell EA. Molnupiravir: coding for catastrophe. Nat Struct Mol Biol. 2021;28:706-708.

280. Mammano F, Petit C, Clavel F. Resistance-associated loss of viral fitness in human immunodeficiency virus type 1: phenotypic analysis of protease and *gag* coevolution in protease inhibitor-treated patients. J Virol. 1998;72:7632-7637.

281. Mansky LM, Temin HM. Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. J Virol. 1995;69:5087-5094.

282. Mar EC, Chiou JF, Cheng YC, Huang ES. Human cytomegalovirus-induced DNA polymerase and its interaction with the triphosphates of 1-(2′-deoxy-2′-fluoro-beta-d-arabinofuranosyl)-5-methyluracil, -5-iodocytosine, and -5-methylcytosine. J Virol. 1985;56:846-851.

283. Marschall M, Marzi A, aus dem Siepen P, et al. Cellular p32 recruits cytomegalovirus kinase pUL97 to redistribute the nuclear lamina. J Biol Chem. 2005;280:33357-33367.

284. Marschall M, Stamminger T, Urban A, et al. In vitro evaluation of the activities of the novel anticytomegalovirus compound AIC246 (letermovir) against herpesviruses and other human pathogenic viruses. Antimicrob Agents Chemother. 2012;56:1135-1137.

285. Martin JL, Brown CE, Matthews-Davis N, Reardon JE. Effects of antiviral nucleoside analogs on human DNA polymerases and mitochondrial DNA synthesis. Antimicrob Agents Chemother. 1994;38:2743-2749.

286. Martin K, Helenius A. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. Cell. 1991;67:117-130.

287. Marty FM, Ljungman P, Chemaly RF, et al. Letermovir prophylaxis for cytomegalovirus in hematopoietic-cell transplantation. N Engl J Med. 2017;377:2433-2444.

288. Marty FM, Ljungman P, Papanicolaou GA, et al. Maribavir prophylaxis for prevention of cytomegalovirus disease in recipients of allogeneic stem-cell transplants: a phase 3, double-blind, placebo-controlled, randomised trial. Lancet Infect Dis. 2011;11:284-292.

289. Marzolini C, Kuritzkes DR, Marra F, et al. Prescribing nirmatrelvir/ritonavir (Paxlovid®): how to recognize and manage drug-drug interactions. Ann Intern Med. 2022;175:744-746.

290. Matsuoka-Aizawa S, Sato H, Hachiya A, et al. Isolation and molecular characterization of a nelfinavir (NFV)-resistant human immunodeficiency virus type 1 that exhibits NFV-dependent enhancement of replication. J Virol. 2003;77:318-327.

291. McClung RP, Oster AM, Ocfemia MCB, et al. Transmitted drug resistance among HIV-1 diagnoses in the United States, 2014-2018. Clin Infect Dis. 2021;74:1055-1062.

292. McColl DJ, Chen X. Strand transfer inhibitors of HIV-1 integrase: bringing IN a new era of antiretroviral therapy. Antiviral Res. 2010;85:101-118.

293. McDaniel KF, Ku Y-Y, Sun Y, et al. The Discovery and Development of HCV NS3 Protease Inhibitor Paritaprevir. Vol 31. Topics in Medicinal Chemistry. Springer; 2019.

294. McKimm-Breschkin JL, Blick TJ, Sahasrabudhe A, et al. Generation and characterization of variants of NWS/G70C influenza virus after in vitro passage in 4-amino-NeuAc2en and 4-guanidino-Neu5Ac2en. Antimicrob Agents Chemother. 1996;40:40-46.

295. Meier A, Mehrle S, Weiss TS, Mier W, Urban S. Myristoylated preS1-domain of the hepatitis B virus L-protein mediates specific binding to differentiated hepatocytes. Hepatology. 2013;58:31-42.

296. Mellors JW, Dutschman GE, Im G-J, Tramontano E, Winkler SR, Cheng Y-C. *In vitro* selection and molecular characterization of human immunodeficiency virus-1 resistant to non-nucleoside inhibitors of reverse transcriptase. Mol Pharmacol. 1992;41:446-451.

297. Memoli MJ, Davis AS, Proudfoot K, et al. Multidrug-resistant 2009 pandemic influenza A(H1N1) viruses maintain fitness and transmissibility in ferrets. J Infect Dis. 2011;203:348-357.

298. Mendel DB, Sidwell RW. Influenza virus resistance to neuraminidase inhibitors. Drug Resist Updat. 1998;1:184-189.

299. Menendez-Arias L. Decoding molnupiravir-induced mutagenesis in SARS-CoV-2. J Biol Chem. 2021;297:100867.

300. Merluzzi VJ, Hargrave KD, Labadia M, et al. Inhibition of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. Science. 1990;250:1411-1413.

301. Metzger C, Michel D, Schneider K, Luske A, Schlicht HJ, Mertens T. Human cytomegalovirus UL97 kinase confers ganciclovir susceptibility to recombinant vaccinia virus. J Virol. 1994;68:8423-8427.

302. Meyer PR, Matsuura SE, So AG, Scott WA. Unblocking of chain-terminated primer by HIV-1 reverse transcriptase through a nucleotide-dependent mechanism. Proc Natl Acad Sci U S A. 1998;95:13471-13476.

303. Meyer PR, Matsuura SE, Tolun AA, et al. Effects of specific zidovudine-resistance mutations and substrate structure on nucleotide-dependent primer unblocking by human immunodeficiency virus type 1 reverse transcriptase. Antimicrob Agents Chemother. 2002;46:1540-1545.

304. Miller V, Phillips AN, Rottman C, et al. Dual resistance to zidovudine and lamivudine in patients treated with zidovudine-lamivudine combination therapy: association with treatment failure. J Infect Dis. 1998;177:398-405.

305. Miller WH, Miller RL. Phosphorylation of acyclovir monophosphate by GMP kinase. J Biol Chem. 1980;255:7204-7207.

306. Miller WH, Miller RL. Phosphorylation of acyclovir diphosphate by cellular enzymes. Biochem Pharmacol. 1982;31:3879-3884.

307. Mitsuya H, Weinhold KJ, Furman PA, et al. 3′-azido-3′-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. Proc Natl Acad Sci U S A. 1985;82:7096-7100.

308. Moore JP, Kuritzkes DR. A pièce de resistance: how HIV-1 escapes small molecule CCR5 inhibitors. Curr Opin HIV AIDS. 2009;4:118-124.

309. Moore MR, Hamzeh FM, Lee FE-H, Lietman PS. Activity of (*S*)-1-(3-hydroxy-2-phosphonylmethoxy)propy)cytosine against human cytomegalovirus when administered as single-bolus dose and continuous infusion in in vitro cell culture perfusion system. Antimicrob Agents Chemother. 1994;38:2404-2408.

310. Moradpour D, Penin F, Rice CM. Replication of hepatitis C virus. Nat Rev Microbiol. 2007;5:453-463.

311. Moss B. Origin of the poxviral membrane: a 50-year old riddle. PLoS Pathog. 2018;14:e1007002.

312. Mozaffari E, Chandak A, Zhang Z, et al. Remdesivir treatment in hospitalized patients with COVID-19: a comparative analysis of in-hospital all-cause mortality in a large multi-center observational cohort. Clin Infect Dis. 2022;75:e450-e458.

313. Murakami E, Niu C, Bao H, et al. The mechanism of action of beta-D-2′-deoxy-2′-fluoro-2′-C-methylcytidine involves a second metabolic pathway leading to beta-D-2′-deoxy-2′-fluoro-2′-C-methyluridine 5′-triphosphate, a potent inhibitor of the hepatitis C virus RNA-dependent RNA polymerase. Antimicrob Agents Chemother. 2008;52: 458-464.

314. Murakami E, Tolstykh T, Bao H, et al. Mechanism of activation of PSI-7851 and its diastereoisomer PSI-7977. J Biol Chem. 2010;285:34337-34347.

315. Nakamura S, Miyazaki T, Izumikawa K, et al. Efficacy and safety of intravenous peramivir compared with oseltamivir in high-risk patients infected with influenza A and B viruses: a multicenter randomized controlled study. Open Forum Infect Dis. 2017;4:ofx129.

316. Navia MA, Fitzgerald PM, McKeever BM, et al. Three-dimensional structure of aspartyl protease from human immunodeficiency virus HIV-1. Nature. 1989;337:615-620.

317. Neurath AR, Kent SBH, Strick N, Parker K. Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. Cell. 1986;46:429-436.

318. Ng TI, Krishnan P, Pilot-Matias T, et al. In vitro antiviral activity and resistance profile of the next-generation hepatitis C virus NS5A inhibitor pibrentasvir. Antimicrob Agents Chemother. 2017;61:e02558-16.

319. Ng TI, Tripathi R, Reisch T, et al. In Vitro Antiviral activity and resistance profile of the next-generation hepatitis C virus NS3/4A protease inhibitor glecaprevir. Antimicrob Agents Chemother. 2018;62:e01620-17.

320. Nguyen HT, Trujillo AA, Sheu TG, et al. Analysis of influenza viruses from patients clinically suspected of infection with an oseltamivir resistant virus during the 2009 pandemic in the United States. Antiviral Res. 2012;93:381-386.

321. Nicholson KG, Aoki FY, Osterhaus AD, et al. Efficacy and safety of oseltamivir in treatment of acute influenza: a randomised controlled trial. Neuraminidase Inhibitor Flu Treatment Investigator Group. Lancet. 2000;355:1845-1850.

322. Noble S, Faulds D. Ganciclovir: an update of its use in the prevention of cytomegalovirus infection and disease in transplant recipients. Drugs. 1998;56:115-146.

323. Noshi T, Kitano M, Taniguchi K, et al. In vitro characterization of baloxavir acid, a first-in-class cap-dependent endonuclease inhibitor of the influenza virus polymerase PA subunit. Antiviral Res. 2018;160:109-117.

324. O'Boyle II DR, Gao M. NS5A as a Target for HCV Drug Discovery. Vol 2. Topics in Medicinal Chemistry. Springer; 2019.

325. O'Boyle II DR, Nower PT, Gao M, et al. Synergistic activity of combined NS5A inhibitors. Antimicrob Agents Chemother. 2015;60:1573-1583.

326. Odriozola L, Cruchaga C, Andréola M, et al. Non-nucleoside inhibitors of HIV-1 reverse transcriptase inhibit phosphorolysis and resensitize the 3′-azido-3′-deoxythymidine (AZT)-resistant polymerase to AZT-5′-triphosphate. J Biol Chem. 2003;278:42710-42716.

327. Ohigashi Y, Ma C, Jing X, Balannick V, Pinto LH, Lamb RA. An amantadine-sensitive chimeric BM2 ion channel of influenza B virus has implications for the mechanism of drug inhibition. Proc Natl Acad Sci U S A. 2009;106:18775-18779.

328. Olender SA, Perez KK, Go AS, et al. Remdesivir for severe coronavirus disease 2019 (COVID-19) versus a cohort receiving standard of care. Clin Infect Dis. 2021;73:e4166-e4174.

329. Olender SA, Walunas TL, Martinez E, et al. Remdesivir versus standard-of-care for severe coronavirus disease 2019 infection: an analysis of 28-day mortality. Open Forum Infect Dis. 2021;8:ofab278.

330. Omoto S, Speranzini V, Hashimoto T, et al. Characterization of influenza virus variants induced by treatment with the endonuclease inhibitor baloxavir marboxil. Sci Rep. 2018;8:9633.

331. Orkin C, Arasteh K, Gorgolas Hernandez-Mora M, et al. Long-acting cabotegravir and rilpivirine after oral induction for HIV-1 infection. N Engl J Med. 2020;382:1124-1135.

332. Ostrander M, Cheng Y-C. Properties of herpes simplex virus type 1 and type 2 DNA polymerase. Biochim Biophys Acta. 1980;609:232-245.

333. Otto MJ, Garber S, Winslow DL, et al. *In vitro* isolation and identification of HIV-1 variants with reduced sensitivity to C2 symmetrical inhibitors of HIV type 1 protease. Proc Natl Acad Sci U S A. 1993;90:7543-7547.

334. Overton ET, Richmond G, Rizzardini G, et al. Long-acting cabotegravir and rilpivirine dosed every 2 months in adults with HIV-1 infection (ATLAS-2M), 48-week results: a randomised, multicentre, open-label, phase 3b, non-inferiority study. Lancet. 2021;396:1994-2005.

335. Owen DR, Allerton CMN, Anderson AS, et al. An oral SARS-CoV-2 M(pro) inhibitor clinical candidate for the treatment of COVID-19. Science. 2021;374:1586-1593.

336. Oxford JS, Potter CW. Aminoadamantane-resistant strains of influenza A2 virus. J Hyg (Lond). 1973;71:227-236.

337. Pahwa S, Biron K, Lim W, et al. Continuous varicella-zoster infection associated with acyclovir resistance in a child with AIDS. JAMA. 1988;260:2879-2882.

338. Painter GR, Natchus MG, Cohen O, Holman W, Painter WP. Developing a direct acting, orally available antiviral agent in a pandemic: the evolution of molnupiravir as a potential treatment for COVID-19. Curr Opin Virol. 2021;50:17-22.

339. Palese P, Compans RW. Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-*N*-trifluoracetylneuraminic acid (FANA): mechanism of action. J Gen Virol. 1976;33:159-163.

340. Palese P, Tobita K, Ueda M, Compans RW. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. Virology. 1974;61:397-410.

341. Pan D, Coen DM. Net -1 frameshifting on a noncanonical sequence in a herpes simplex virus drug-resistant mutant is stimulated by nonstop mRNA. Proc Natl Acad Sci U S A. 2012;109:14852-14857.

342. Pancera M, Lai YT, Bylund T, et al. Crystal structures of trimeric HIV envelope with entry inhibitors BMS-378806 and BMS-626529. Nat Chem Biol. 2017;13:1115-1122.

343. Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the Use of Antiretroviral Agents in Adults and Adolescents with HIV. Department of Health and Human Services; 2022.

344. Penin F, Brass V, Appel N, et al. Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A. J Biol Chem. 2004;279(39): 40835-40843.

345. Perelson AS, Layden TJ. Ribavirin: is it a mutagen for hepatitis C virus? Gastroenterology. 2007;132:2050-2052.

346. Perni RB, Almquist SJ, Byrn RA, et al. Preclinical profile of VX-950, a potent, selective, and orally bioavailable inhibitor of hepatitis C virus NS3-4A serine protease. Antimicrob Agents Chemother. 2006;50:899-909.

347. Petit SC, Simsic J, Loeb DD, Everitt L, Hutchison CA III, Swanstrom R. Analysis of retroviral protease cleavage sites reveals two types of cleavage sites and the structural requirements of the P1 amino acid. J Biol Chem. 1991;266:14539-14547.

348. Pfeiffer JK, Kirkegaard K. Ribavirin resistance in hepatitis C virus replicon-containing cell lines conferred by changes in the cell line or mutations in the replicon RNA. J Virol. 2005;79:2346-2355.

349. Pfizer. Fact Sheet for Healthcare Providers: Emergency Use Authorization of Paxlovid®. 2022.

350. Phan T, Fay EJ, Lee Z, Aron S, Hu WS, Langlois RA. Segment-specific kinetics of mRNA, cRNA and vRNA accumulation during influenza infection. J Virol. 2021;95:e02102-e02120.

351. Pianko S, Flamm SL, Shiffman ML, et al. Sofosbuvir plus velpatasvir combination therapy for treatment-experienced patients with genotype 1 or 3 hepatitis C virus infection: a randomized trial. Ann Intern Med. 2015;163:809-817.

352. Pinto LH, Holsinger LJ, Lamb RA. Influenza virus M2 protein has ion channel activity. Cell. 1992;69:517-528.

353. Plotch SJ, Bouloy M, Ulmanen I, Krug RM. A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell. 1981;23:847-858.

354. Poorman RA, Tomaselli AG, Heinrikson RL, Kezdy FJ. A cumulative specificity model for proteases from human immunodeficiency virus types 1 and 2 inferred from statistical analysis of an extended substrate data base. J Biol Chem. 1991;266:14554-14561.

355. Prichard MN, Gao N, Jairath S, et al. A recombinant human cytomegalovirus with a large deletion in UL97 has a severe replication deficiency. J Virol. 1999;73:5663-5670.

356. Prichard MN, Sztul E, Daily SL, et al. Human cytomegalovirus UL97 kinase is required for the hyperphosphorylation of retinoblastoma protein and inhibits the formation of nuclear aggresomes. J Virol. 2008;82:5054-5067.

357. Pulley S. CCR5 antagonists: from discovery to clinical efficacy. In: Neote K, Letts GL, Moser B, eds. Chemokine Biology — Basic Research and Clinical Application. Vol 2. Birkhauser; 2007:145-163.

358. Rameix-Welti MA, Enouf V, Cuvelier F, Jeannin P, van der Werf S. Enzymatic properties of the neuraminidase of seasonal H1N1 influenza viruses provide insights for the emergence of natural resistance to oseltamivir. PLoS Pathog. 2008;4:e1000103.

359. Randomised trial of efficacy and safety of inhaled zanamivir in treatment of influenza A and B virus infections. The MIST (Management of Influenza in the Southern Hemisphere Trialists) Study Group. Lancet. 1998;352:1877-1881.

360. Reardon JE. Herpes simplex virus type 1 and human DNA polymerase interactions with 2′-deoxyguanosine-5′-triphosphate analogues. J

Biol Chem. 1989;264:19039-19044.

361. Reardon JE, Spector T. Herpes simplex virus type 1 DNA polymerase. Mechanism of inhibition by acyclovir triphosphate. J Biol Chem. 1989;264:7405-7411.

362. Rebsamen M, Meylan E, Curran J, Tschopp J. The antiviral adaptor proteins Cardif and Trif are processed and inactivated by caspases. Cell Death Differ. 2008;15: 1804-1811.

363. Reid R, Mar EC, Huang ES, Topal MD. Insertion and extension of acyclic, dideoxy, and ara nucleotides by herpesviridae, human alpha and human beta polymerases. A unique inhibition mechanism for 9-(1,3-dihydroxy-2-propoxymethyl)guanine triphosphate. J Biol Chem. 1988;263:3898-3904.

364. Reim NI, Kamil JP, Wang D, et al. Inactivation of retinoblastoma protein does not overcome the requirement for human cytomegalovirus UL97 in lamina disruption and nuclear egress. J Virol. 2013;87:5019-5027.

365. Reitsma JM, Savaryn JP, Faust K, Sato H, Halligan BD, Terhune SS. Antiviral inhibition targeting the HCMV kinase pUL97 requires pUL27-dependent degradation of Tip60 acetyltransferase and cell-cycle arrest. Cell Host Microbe. 2011;9:103-114.

366. REMAP-CAP Investigators; Gordon AC, Mouncey PR, et al. Interleukin-6 receptor antagonists in critically ill patients with covid-19. N Engl J Med. 2021;384:1491-1502.

367. Richman D, Rosenthal AS, Skoog M, et al. BI-RG-587 is active against zidovudine-resistant human immunodeficiency virus type 1 and synergistic with zidovudine. Antimicrob Agents Chemother. 1991;35:305-308.

368. Richman D, Shih C-K, Lowy I, et al. Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. Proc Natl Acad Sci U S A. 1991;88:11241-11245.

369. Richman DD. Antiretroviral activity of emtricitabine, a potent nucleoside reverse transcriptase inhibitor. Antivir Ther. 2001;6:83-88.

370. Richman DD, Fischl MA, Grieco MH, et al. The toxicity of 3′-azido-3′deoxythymidine (azidothymidine) in the treatment of patients with ADIS and AIDS-related complex. N Engl J Med. 1987;317:192-197.

371. Richman DD, Havlir D, Corbeil J, et al. Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. J Virol. 1994;68:1660-1666.

372. Rimsky LT, Shugars DC, Matthews TJ. Determinants of human immunodeficiency virus type 1 resistance to gp41-derived peptides. J Virol. 1998;72:986-993.

373. Rittinger K, Divita G, Goody RS. Human immunodeficiency virus reverse transcriptase substrate-induced conformational changes and the mechanism of inhibition by nonnucleoside inhibitors. Proc Natl Acad Sci U S A. 1995;92:8046-8049.

374. Rodriguez-Torres M. Sofosbuvir (GS-7977), a pan-genotype, direct-acting antiviral for hepatitis C virus infection. Expert Rev Anti Infect Ther. 2013;11:1269-1279.

375. Rogers DW, Gamblin SJ, Harris BA, et al. The structure of unliganded reverse transcriptase from human immunodeficiency virus type 1. Proc Natl Acad Sci U S A. 1995;92: 1222-1226.

376. Ronn R, Sabnis YA, Gossas T, et al. Exploration of acyl sulfonamides as carboxylic acid replacements in protease inhibitors of the hepatitis C virus full-length NS3. Bioorg Med Chem. 2006;14:544-559.

377. Roosenhoff R, Reed V, Kenwright A, et al. Viral kinetics and resistance development in children treated with neuraminidase inhibitors: the Influenza Resistance Information Study (IRIS). Clin Infect Dis. 2020;71:1186-1194.

378. Roper RL, Moss B. Envelope formation is blocked by mutation of a sequence related to the HKD phospholipid metabolism motif in the vaccinia virus F13L protein. J Virol. 1999;73:1108-1117.

379. Rosen HR. Clinical practice. Chronic hepatitis C infection. N Engl J Med. 2011;364: 2429-2438.

380. Rosenquist A, Samuelsson B, Johansson PO, et al. Discovery and development of simeprevir (TMC435), a HCV NS3/4A protease inhibitor. J Med Chem. 2014;57:1673-1693.

381. Ross-Thriepland D, Harris M. Hepatitis C virus NS5A: enigmatic but still promiscuous 10 years on! J Gen Virol. 2015;96:727-738.

382. Russo AT, Grosenbach DW, Chinsangaram J, et al. An overview of tecovirimat for smallpox treatment and expanded anti-orthopoxvirus applications. Expert Rev Anti Infect Ther. 2021;19:331-344.

383. Saag MS, Gandhi RT, Hoy JF, et al. Antiretroviral drugs for treatment and prevention of HIV infection in adults: 2020 recommendations of the International Antiviral Society-USA Panel. JAMA. 2020;324:1651-1669.

384. Safrin S, Crumpacker C, Chatis P, et al. A controlled trial comparing foscarnet with vidarabine for acyclovir-resistant mucocutaneous herpes simplex in the acquired immunodeficiency syndrome. The AIDS Clinical Trials Group. N Engl J Med. 1991;325:551-555.

385. Safrin S, Kemmerly S, Plotkin B, et al. Foscarnet-resistant herpes simplex virus infection in patients with AIDS. J Infect Dis. 1994;169:193-196.

386. Sarafianos SG, Das K, Clark AD Jr, et al. Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with β-branched amino acids. Proc Natl Acad Sci U S A. 1999;96:10027-10032.

387. Sarisky RT, Quail MR, Clark PE, et al. Characterization of herpes simplex viruses selected in culture for resistance to penciclovir or acyclovir. J Virol. 2001;75:1761-1769.

388. Sarrazin C, Kieffer TL, Bartels D, et al. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. Gastroenterology. 2007;132:1767-1777.

389. Schaeffer HJ, Beauchamp L, de Miranda P, Elion GB, Bauer DJ, Collins P. 9-(2-hydroxyethoxymethyl)guanine activity against viruses of the herpes group. Nature. 1978;272:583-585.

390. Scheidel LM, Durbin RK, Stollar V. Sindbis virus mutants resistant to mycophenolic acid and ribavirin. Virology. 1987;158:1-7.

391. Schinazi RF, Chu CK, Peck A, et al. Activities of the four optical isomers of 2'-3'-dideoxy-3'-thiacytidine (BCH-189) against human immunodeficiency virus type 1 in human lymphocytes. Antimicrob Agents Chemother. 1992;36:672-676.

392. Seifer M, Hamatake RK, Colonno RJ, Standring DN. In vitro inhibition of hepadnavirus polymerases by the triphosphates of BMS-200475 and lobucavir. Antimicrob Agents Chemother. 1998;42:3200-3208.

393. Seifert M, Bera SC, van Nies P, et al. Inhibition of SARS-CoV-2 polymerase by nucleotide analogs from a single-molecule perspective. Elife. 2021;10:e70968.

394. Selmi B, Deval J, Alvarez K, et al. The Y181C substitution in 3′-azido-3′-deoxythymidine-resistant human immunodeficiency virus type 1 reverse transcriptase suppresses the ATP-mediated repair of the 3′-azido-3′-deoxythymidine 5'-monophosphate-terminated primer. J Biol Chem. 2003;278:40464-40472.

395. Shannon-Lowe CD, Emery VC. The effects of maribavir on the autophosphorylation of ganciclovir resistant mutants of the cytomegalovirus UL97 protein. Herpesviridae. 2010;1:4.

396. Sharma M, Bender BJ, Kamil JP, et al. Human cytomegalovirus phosphorylates the viral nuclear egress complex. J Virol. 2015;89:523-534.

397. Sheahan TP, Sims AC, Zhou S, et al. An orally bioavailable broad-spectrum antiviral inhibits SARS-CoV-2 in human airway epithelial cell cultures and multiple coronaviruses in mice. Sci Transl Med. 2020;12:eabb5883.

398. Sheikh AY, Mattei A, Miglani Bhardwaj R, et al. Implications of the conformationally flexible, macrocyclic structure of the first-generation, direct-acting anti-viral paritaprevir on its solid form complexity and chameleonic behavior. J Am Chem Soc. 2021;143:17479-17491.

399. Shirasaka T, Yarchoan R, O'Brien MC, et al. Changes in drug sensitivity of human immunodeficiency virus type 1 during therapy with azidothymidine, dideoxycytidine, and dideoxyinosine: An in vitro comparative study. Proc Natl Acad Sci U S A. 1993;90: 562-566.

400. Si Z, Madani N, Cox JM, et al. Small-molecule inhibitors of HIV-1 entry block receptor-induced conformational changes in the viral envelope glycoproteins. Proc Natl Acad Sci U S A. 2004;101:5036-5041.

401. Siddiqi HK, Mehra MR. COVID-19 illness in native and immunosuppressed states: a clinical-therapeutic staging proposal. J Heart Lung Transplant. 2020;39:405-407.

402. Siegel D, Hui HC, Doerffler E, et al. Discovery and synthesis of a phosphoramidate prodrug of a pyrrolo[2,1-f][triazin-4-amino] adenine C-nucleoside (GS-5734) for the treatment of ebola and emerging viruses. J Med Chem. 2017;60:1648-1661.

403. Silva LA, Strang BL, Lin EW, Kamil JP, Coen DM. Sites and roles of phosphorylation of the human cytomegalovirus DNA polymerase subunit UL44. Virology. 2011;417: 268-280.

404. Silva MO, Treitel M, Graham DJ, et al. Antiviral activity of boceprevir monotherapy in treatment-naive subjects with chronic hepatitis C genotype 2/3. J Hepatol. 2013;59:31-37.

405. Slain D. Intravenous zanamivir: a viable option for critically ill patients with influenza. Ann Pharmacother. 2021;55:760-771.

406. Snow-Lampart A, Chappell B, Curtis M, et al. No resistance to tenofovir disoproxil fumarate detected after up to 144 weeks of therapy in patients monoinfected with chronic hepatitis B virus. Hepatology. 2011;53:763-773.

407. Sofia MJ, Bao D, Chang W, et al. Discovery of a beta-d-2′-deoxy-2′-alpha-fluoro-2′-beta-C-methyluridine nucleotide prodrug (PSI-7977) for the treatment of hepatitis C virus. J Med Chem. 2010;53:7202-7218.

408. Spence RA, Kati WM, Anderson KS, Johnson KA. Mechanism of inhibition of HIV-1 reverse transcriptase. Science. 1995;267:988-993.

409. Spinner CD, Gottlieb RL, Criner GJ, et al. Effect of remdesivir vs standard care on clinical status at 11 days in patients with moderate COVID-19: a randomized clinical trial. JAMA. 2020;324:1048-1057.

410. St Clair MH, Miller WH, Miller RL, Lambe CU, Furman PA. Inhibition of cellular α DNA polymerase and herpes simplex virus-induced DNA polymerases by the triphosphate of BW759U. Antimicrob Agents Chemother. 1984;25:191-194.

411. St Clair MH, Richards CA, Spector T, et al. 3′-azido-3′-deoxythymidine triphosphate as an inhibitor and substrate of purified human immunodeficiency virus reverse transcriptase. Antimicrob Agents Chemother. 1987;31:1972-1977.

412. Staschke KA, Colacino JM, Baxter AJ, et al. Molecular basis for the resistance of influenza viruses to 4-guanidino-Neu5Ac2en. Virology. 1995;214:642-646.

413. Stevens LJ, Pruijssers AJ, Lee HW, et al. Mutations in the SARS-CoV-2 RNA dependent RNA polymerase confer resistance to remdesivir by distinct mechanisms. Sci Transl Med. 2022;14:eabo0718.

414. Stouffer AL, Acharya R, Salom D, et al. Structural basis for the function and inhibition of an influenza virus proton channel. Nature. 2008;451:596-599.

415. Sugrue RJ, Hay AJ. Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel. Virology. 1991;180:617-624.

416. Sulkowski MS, Algahtani SA. The Role of Interferon for the Treatment of Chronic Hepatitis C Virus Infection. Vol 31. Topics in Medicinal Chemistry. Springer; 2019.

417. Sullivan V, Talarico CL, Stanat SC, Davis M, Coen DM, Biron KK. A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. Nature. 1992;358:162-164.

418. Summa V, Petrocchi A, Bonelli F, et al. Discovery of raltegravir, a potent, selective orally bioavailable HIV-integrase inhibitor for the treatment of HIV-AIDS infection. J Med Chem. 2008;51:5843-5855.

419. Sun JH, O'Boyle DR II, Fridell RA, et al. Resensitizing daclatasvir-resistant hepatitis C variants by allosteric modulation of NS5A. Nature. 2015;527:245-248.

420. Sung TC, Roper RL, Zhang Y, et al. Mutagenesis of phospholipase D defines a superfamily including a trans-Golgi viral protein required for poxvirus pathogenicity. EMBO J. 1997;16:4519-4530.

421. Suppiah V, Moldovan M, Ahlenstiel G, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet. 2009;41:1100-1104.

422. Sureau C, Guerra B, Lanford RE. Role of the large hepatitis B virus envelope protein in the infectivity of the hepatitis delta virion. J Virol. 1993;67:366-372.

423. Susser S, Vermehren J, Forestier N, et al. Analysis of long-term persistence of resistance mutations within the hepatitis C virus NS3 protease after treatment with telaprevir or boceprevir. J Clin Virol. 2011;52:321-327.

424. Suzuki Y, Kumada H, Ikeda K, et al. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J Hepatol. 1999;30:743-758.

425. Suzutani T, Ishioka K, De Clercq E, et al. Differential mutation patterns in thymidine kinase and DNA polymerase genes of herpes simplex virus type 1 clones passaged in the presence of acyclovir and penciclovir. Antimicrob Agents Chemother. 2003;47:1707-1713.

426. Svarovskaia ES, Dvory-Sobol H, Parkin N, et al. Infrequent development of resistance in genotype 1-6 hepatitis C virus-infected subjects treated with sofosbuvir in phase 2 and 3 clinical trials. Clin Infect Dis. 2014;59:1666-1674.

427. Sweet C, Hayden FG, Jakeman KJ, Grambas S, Hay AJ. Virulence of rimantadine-resistant human influenza A (H3N2) viruses in ferrets. J Infect Dis. 1991;164:969-972.

428. Swindells S, Andrade-Villanueva JF, Richmond GJ, et al. Long-acting cabotegravir and rilpivirine for maintenance of HIV-1 suppression. N Engl J Med. 2020;382:1112-1123.

429. Szemiel AM, Merits A, Orton RJ, et al. In vitro selection of remdesivir resistance suggests evolutionary predictability of SARS-CoV-2. PLoS Pathog. 2021;17:e1009929.

430. Takashita E, Kinoshita N, Yamayoshi S, et al. Efficacy of antibodies and antiviral drugs against covid-19 omicron variant. N Engl J Med. 2022;386:995-998.

431. Talarico CL, Burnette TC, Miller WH, et al. Acyclovir is phosphorylated by the human cytomegalovirus UL97 protein. Antimicrob Agents Chemother. 1999;43:1941-1946.

432. Tan K, Liu JH, Want JH, Shen S, Lu M. Atomic structure of a thermostable subdomain of HIV-1 gp41. Proc Natl Acad Sci U S A. 1997;94:12303-12308.

433. Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet. 2009;41:1105-1109.

434. Taylor JG. Discovery of Voxilaprevir (GS-9857): The Pan-Genotypic Hepatitis C Virus NS3/4A Protease Inhibitor Utilized as a Component of Vosevi®. Vol 31. Topics in Medicinal Chemistry. Springer; 2019.

435. Tchesnokov EP, Gordon CJ, Woolner E, et al. Template-dependent inhibition of coronavirus RNA-dependent RNA polymerase by remdesivir reveals a second mechanism of action. J Biol Chem. 2020;295:16156-16165.

436. Te Velthuis AJ, Fodor E. Influenza virus RNA polymerase: insights into the mechanisms of viral RNA synthesis. Nat Rev Microbiol. 2016;14:479-493.

437. Tellinghuisen TL, Marcotrigiano J, Rice CM. Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. Nature. 2005;435:374-379.

438. Terrault NA, Lok ASF, McMahon BJ, et al. Update on prevention, diagnosis, and treatment of chronic hepatitis B: AASLD 2018 hepatitis B guidance. Hepatology. 2018;67: 1560-1599.

439. The studies of ocular complications of AIDS research group in collaboration with the AIDS clinical trials group. Combination foscarnet and ganciclovir therapy vs. monotherapy for the treatment of relapsed cytomegalovirus retinitis in patients with AIDS. Arch Ophthalmol. 1996;114:23-33.

440. Todd B, Tchesnokov EP, Gotte M. The active form of the influenza cap-snatching endonuclease inhibitor baloxavir marboxil is a tight binding inhibitor. J Biol Chem. 2021;296:100486.

441. Treanor JJ, Hayden FG, Vrooman PS, et al. Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza: a randomized controlled trial. US Oral Neuraminidase Study Group. JAMA. 2000;283:1016-1024.

442. Trkola A, Kuhmann SE, Strizki JM, et al. HIV-1 escape from a small molecule, CCR5-specific entry inhibitor does not involve CXCR4 use. Proc Natl Acad Sci U S A. 2002;99: 395-400.

443. Tu X, Das K, Han Q, et al. Structural basis of HIV-1 resistance to AZT by excision. Nat Struct Mol Biol. 2010;17:1202-1209.

444. Turner TL, Kopp BT, Paul G, Landgrave LC, Hayes D Jr, Thompson R. Respiratory syncytial virus: current and emerging treatment options. Clinicoecon Outcomes Res. 2014;6: 217-225.

445. Tyring S, Barbarash RA, Nahlik JE, et al. Famciclovir for the treatment of acute herpes zoster: effects on acute disease and postherpetic neuralgia. A randomized, double-blind, placebo-controlled trial. Collaborative Famciclovir Herpes Zoster Study Group. Ann Intern Med. 1995;123:89-96.

446. V'Kovski P, Kratzel A, Steiner S, Stalder H, Thiel V. Coronavirus biology and replication: implications for SARS-CoV-2. Nat Rev Microbiol. 2021;19:155-170.

447. Vangeel L, Chiu W, De Jonghe S, et al. Remdesivir, molnupiravir and nirmatrelvir remain active against SARS-CoV-2 Omicron and other variants of concern. Antiviral Res. 2022;198:105252.

448. Varghese JN, McKimm-Breschkin JL, Caldwell JB, Kortt AA, Colman PM. The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. Proteins. 1992;14:327-332.

449. Vaz FM, Paulusma CC, Huidekoper H, et al. Sodium taurocholate cotransporting polypeptide (SLC10A1) deficiency: conjugated hypercholanemia without a clear clinical phenotype. Hepatology. 2015;61:260-267.

450. Venkatraman S, Bogen SL, Arasappan A, et al. Discovery of (1R,5S)-N-[3-amino-1-(cyclobutylmethyl)-2,3-dioxopropyl]- 3-[2(S)-[[[(1,1-dimethylethyl)amino]carbonyl]amino]-3,3-dimethyl-1-oxobutyl]- 6,6-dimethyl-3-azabicyclo[3.1.0]hexan-2(S)-carboxamide (SCH 503034), a selective, potent, orally bioavailable hepatitis C virus NS3 protease inhibitor: a potential therapeutic agent for the treatment of hepatitis C infection. J Med Chem. 2006;49:6074-6086.

451. Verbinnen T, Fevery B, Vijgen L, Jacobs T, De Meyer S, Lenz O. In vitro activity of simeprevir against hepatitis C virus genotype 1 clinical isolates and its correlation with NS3 sequence and site-directed mutants. Antimicrob Agents Chemother. 2015;59:7548-7557.

452. von Itzstein M, Wu WY, Kok GB, et al. Rational design of potent sialidase-based inhibitors of influenza virus replication. Nature. 1993;363:418-423.

453. Vora S, Damon I, Fulginiti V, et al. Severe eczema vaccinatum in a household contact of a smallpox vaccinee. Clin Infect Dis. 2008;46:1555-1561.

454. Wahl A, Gralinski LE, Johnson CE, et al. SARS-CoV-2 infection is effectively treated and prevented by EIDD-2801. Nature. 2021;591:451-457.

455. Wang M, Cao R, Zhang L, et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. Cell Res. 2020;30: 269-271.

456. Wang Q, Wu J, Wang H, et al. Structural basis for RNA replication by the SARS-CoV-2 polymerase. Cell. 2020;182:417-428 e13.

457. Warren TK, Jordan R, Lo MK, et al. Therapeutic efficacy of the small molecule GS-5734 against Ebola virus in rhesus monkeys. Nature. 2016;531:381-385.

458. Weissenhorn W, Dessen A, Harrison SC, Skehel JJ, Wiley DC. Atomic structure of the ectodomain from HIV-1 gp41. Nature. 1997;387:426-430.

459. Wensing AM, Calvez V, Ceccherini-Silberstein F, et al. 2019 update of the drug resistance mutations in HIV-1. Top Antivir Med. 2019;27:111-121.

460. Westby M, Smith-Burchnell C, Mori J, et al. Reduced maximal inhibition in phenotypic susceptibility assays indicates that viral strains resistant to the CCR5 antagonist maraviroc utilize inhibitor-bound receptor for entry. J Virol. 2007;81:2359-2371.

461. Wetherall NT, Trivedi T, Zeller J, et al. Evaluation of neuraminidase enzyme assays using different substrates to measure susceptibility of influenza virus clinical isolates to neuraminidase inhibitors: report of the neuraminidase inhibitor susceptibility network. J Clin Microbiol. 2003;41:742-750.

462. Whitcomb JM, Parkin NT, Chappey C, Hellmann NS, Petropoulos CJ. Broad nucleoside reverse transcriptase inhibitor cross-resistance in human immunodeficiency virus type 1 clinical isolates. J Infect Dis. 2003;188:992-1000.

463. Whitley RJ, Gnann JW Jr. Acyclovir: a decade later. N Engl J Med. 1992;327:782-789.

464. WHO Solidarity Trial Consortium, Pan H, Peto R, et al. Repurposed antiviral drugs for covid-19—interim WHO solidarity trial results. N Engl J Med. 2021;384:497-511.

465. Wild C, Dubay J, Greenwell TK, et al. Propensity for a leucine zipper-like domain of human immunodeficiency virus type 1 gp41 to form oligomers correlates with a role in virus-induced fusion rather than assembly of the glycoprotein complex. Proc Natl Acad Sci U S A.

1994;91:12676-12680.

466. Wild C, Greenwell T, Shugars D, Rimsky-Clarke L, Matthews T. The inhibitory activity of an HIV type 1 peptide correlates with its ability to interact with a leucine zipper structure. AIDS Res Hum Retroviruses. 1995;11:323-325.

467. Wild C, Oas T, McDanal C, Bolognesi D, Matthews TR. A synthetic peptide inhibitor of HIV replication: correlation between solution structure and viral inhibition. Proc Natl Acad Sci U S A. 1992;89:10537-10541.

468. Wild C, Shugars DC, Greenwell TK, McDanal CB, Matthews TJ. Peptides corresponding to a predictive alpha-helical domain of HIV-1 gp41 are potent inhibitors of virus infection. Proc Natl Acad Sci U S A. 1994;91:9770-9774.

469. Wild K, Bohner T, Aubry A, Folkers G, Schulz GE. The structures of thymidine kinase from herpes simplex virus type 1 complexed with substrates and a substrate analogue. Protein Sci. 1997;6:2097-2106.

470. Williamson BN, Feldmann F, Schwarz B, et al. Clinical benefit of remdesivir in rhesus macaques infected with SARS-CoV-2. Nature. 2020;585:273-276.

471. Winston DJ, Young JA, Pullarkat V, et al. Maribavir prophylaxis for prevention of cytomegalovirus infection in allogeneic stem cell transplant recipients: a multicenter, randomized, double-blind, placebo-controlled, dose-ranging study. Blood. 2008;111:5403-5410.

472. Wlodawer A, Erickson JW. Structure based inhibitors of HIV-1 protease. Annu Rev Biochem. 1993;62:543-580.

473. Wolf DG, Courcelle CT, Prichard MN, Mocarski ES. Distinct and separate roles for herpesvirus-conserved UL97 kinase in cytomegalovirus DNA synthesis and encapsidation. Proc Natl Acad Sci U S A. 2001;98:1895-1900.

474. Wong KA, Worth A, Martin R, et al. Characterization of hepatitis C virus resistance from a multiple-dose clinical trial of the novel NS5A inhibitor GS-5885. Antimicrob Agents Chemother. 2013;57:6333-6340.

475. Woo G, Tomlinson G, Nishikawa Y, et al. Tenofovir and entecavir are the most effective antiviral agents for chronic hepatitis B: a systematic review and Bayesian meta-analyses. Gastroenterology. 2010;139:1218-1229.

476. World Health Organization. HIV Drug Resistance Report 2019. World Health Organization; 2019.

477. World Health Organization. Update of recommendations on first- and second-line antiretroviral regimens. World Health Organization; 2019.

478. Wu JC, Warren TC, Adams J, et al. A novel dipyridodiazepinone inhibitor of HIV-1 reverse transcriptase acts through a nonsubstrate binding site. Biochemistry. 1991;30:2022-2026.

479. Xiong X, Smith JL, Chen MS. Effect of incorporation of cidofovir into DNA by human cytomegalovirus DNA polymerase on DNA elongation. Antimicrob Agents Chemother. 1997;41:594-599.

480. Xiong X, Smith JL, Kim CU, Huang E-S, Chen MS. Kinetic analysis of the interaction of cidofovir diphosphate with human cytomegalovirus DNA polymerase. Biochem Pharmacol. 1996;51:1563-1567.

481. Yamashita M, Engelman AN. Capsid-dependent host factors in HIV-1 infection. Trends Microbiol. 2017;25:741-755.

482. Yan H, Zhong G, Xu G, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife. 2012;1:e00049.

483. Yang G, Pevear DC, Davies MH, et al. An orally bioavailable antipoxvirus compound (ST-246) inhibits extracellular virus formation and protects mice from lethal orthopoxvirus Challenge. J Virol. 2005;79:13139-13149.

484. Yant SR, Mulato A, Hansen D, et al. A highly potent long-acting small-molecule HIV-1 capsid inhibitor with efficacy in a humanized mouse model. Nat Med. 2019;25:1377-1384.

485. Yoshino R, Yasuo N, Sekijima M. Molecular dynamics simulation reveals the mechanism by which the influenza cap-dependent endonuclease acquires resistance against baloxavir marboxil. Sci Rep. 2019;9:17464.

486. Young D, Fowler C, Bush K. RWJ-270201 (BCX-1812): a novel neuraminidase inhibitor for influenza. Philos Trans R Soc Lond B Biol Sci. 2001;356:1905-1913.

487. Zahn KE, Tchesnokov EP, Gotte M, Doublie S. Phosphonoformic acid inhibits viral replication by trapping the closed form of the DNA polymerase. J Biol Chem. 2011;286:25246-25255.

488. Zandi K, Amblard F, Musall K, et al. Repurposing nucleoside analogs for human coronaviruses. Antimicrob Agents Chemother. 2020;65:e01652-20.

489. Zennou V, Mammano F, Paulous S, Mathez D, Clavel F. Loss of viral fitness associated with multiple Gag and Gag-Pol processing defects in human immunodeficiency virus type 1 variants selected for resistance to protease inhibitors in vivo. J Virol. 1998;72:3300-3306.

490. Zeuzem S, Foster GR, Wang S, et al. Glecaprevir-Pibrentasvir for 8 or 12 weeks in HCV genotype 1 or 3 infection. N Engl J Med. 2018;378:354-369.

491. Zeuzem S, Ghalib R, Reddy KR, et al. Grazoprevir-Elbasvir combination therapy for treatment-naive cirrhotic and noncirrhotic patients with chronic hepatitis C virus genotype 1, 4, or 6 infection: a randomized trial. Ann Intern Med. 2015;163:1-13.

492. Zhou N, Han Z, Hartman-Neumann S, et al. Characterization of NS5A polymorphisms and their impact on response rates in patients with

HCV genotype 2 treated with daclatasvir-based regimens. J Antimicrob Chemother. 2016;71:3495-3505.

CHAPTER 15

Immunization Against Viral Diseases

James E. Crowe Jr

History of viral vaccine development and future prospects

Origins of vaccinology

Critical events in the history of viral vaccines

Goals of immunization against viral diseases

Prevention of disease or infection

Immunotherapy

Eradication or elimination

How vaccines work

Correlates of protection

Mechanisms of vaccine-induced immunity

Vaccine-induced cellular immunity

Obstacles to immunization in special populations

Immunization of infants

Immunization of the elderly

Immunization of pregnant women

Immunization of the immunocompromised

New conceptual approaches to rational vaccine design—structure-based vaccines

Advances in monoclonal Ab isolation

Advances in structural biology coupled with computational biology

Types of vaccines

Live virus vaccines

Inactivated virus vaccines

Viruslike particle vaccines

Disadvantages of viruslike particle vaccines

Other vaccine approaches

Vaccination by gene delivery

Vaccine formulation and delivery

Adjuvants

Delivery vehicles

Combination approaches

Summary

Acknowledgments

Both innate and adaptive immune responses mediate host resistance to virus infection. Elements of the innate immune system, especially type I interferons, strongly influence the clinical outcome of a virus infection. Many viruses have evolved mechanisms to inhibit or evade innate immune responses, indicating the importance of those defenses in limiting virus infection. Innate immune responses also mediate important effects during the induction phase of an immune response and may influence the magnitude and composition of adaptive immune responses. The capacity for somatic diversification of B cell receptors and selection and long-term memory for B and T cells are central properties of

adaptive immunity. Therefore, vaccine-induced immunity that is established in advance of virus infection relies primarily on adaptive immune responses for protective efficacy. Vaccination depends critically on the properties of Ag recognition, activation, expansion, memory, trafficking, and effector functions inherent to lymphocytes. Complex influences of vaccination on the timing, magnitude, specificity, quality, and location of responding lymphocytes and their effector molecules determine whether exposure to a viral pathogen results in infection, protection, subclinical infection, mild disease, or exaggerated disease expression. The extent to which vaccine-induced immunity is successful in an individual can determine the spread of virus within a population (e-Fig. 15.1). Thus, vaccines have a profound effect on public health through the control of epidemics (e-Fig. 15.2). An antigenic stimulus that elicits a virus-specific adaptive immune response that can be recalled during subsequent virus infection defines it as an immunization. A vaccination is an antigenic stimulus delivered by intention. Natural exposures to circulating viruses (in vaccinated or antigen-naive individuals) also can induce immunity and may contribute to establishing or maintaining population immunity, even if the resulting infections are subclinical.

e-FIGURE 15.1 **Points where vaccination can interrupt the dynamics of viral spread in an individual and the population.** Vaccination of an individual not only provides direct benefit by producing immunity to prevent disease but also has a profound indirect effect on public health by interrupting the cycle of transmission. As transmitters and susceptible hosts are removed from a population, the spread of infection becomes less likely. To be successful, a virus must reach a susceptible host, attach to a susceptible cell, enter that cell, and evade clearance by host defenses long enough to be transmitted to another susceptible host. Each step in the process represents an opportunity for vaccine-induced immune responses to limit spread of the virus.

e-FIGURE 15.2 **Vaccine-mediated control of epidemic viral infection.** The spread of infectious diseases within a population can be described largely by (1) transmission efficiency, (2) rates of contact between infected and susceptible people, and (3) duration of viral shedding or contagiousness of the transmitter. These factors support the proverbial 3-legged stool that represents the expansion or contraction of an epidemic, and each is influenced by different aspects of vaccine-mediated immunity.

History of Viral Vaccine Development and Future Prospects

The history of discovery and development for each viral vaccine is unique and instructional. Although space limitations prevent the full description of each vaccine, some of the major events are outlined below. Although each vaccine development story is different, there are common events and themes that have been catalogued in a supplementary table (e-Table 15.1) that includes the following: (1) disease description and virus discovery, (2) vaccine discovery and/or proof-of-concept, (3) vaccine licensure or first human use, and (4) unique or unanticipated events.

e-TABLE 15.1 History of selected viral vaccine development

[1]Huebner RJ, Rowe WP, Ward TG, et al. Adenoidal-pharyngeal-conjunctival agents: a newly recognized group of common viruses of the respiratory system. *N Engl J Med*. 1954;251(27):1077-1086.

[2]Rowe WP, Huebner RJ, Bell JA. Definition and outline of contemporary information on the adenovirus group. *Ann N Y Acad Sci*. 1957;67(8):255-261.

[3]Hilleman MR. Epidemiology of adenovirus respiratory infections in military recruit populations. *Ann N Y Acad Sci*. 1957;67(8):262-272.

[4]Trentin JJ, Yabe Y, Taylor G. The quest for human cancer viruses. *Science*. 1962;137:835-841.

[5]Gray GC, Goswami PR, Malasig MD, et al. Adult adenovirus infections: loss of orphaned vaccines precipitates military respiratory disease epidemics. For the Adenovirus Surveillance Group. *Clin Infect Dis*. 2000;31(3):663-670.

[6]Gaydos CA, Gaydos JC. Adenovirus vaccines. In: Orenstein PA, ed. *Vaccines*. 4th ed. Saunders; 2004:863-885.

[7]Moritz S. Epidemic jaundice in war time. *Br Med J*. 1915;2(2860):602.

[8]Maccallum FO, Bradley WH. Transmission of infective hepatitis to human volunteers. *Lancet*. 1944;244(6311):228.

[9]Maccallum FO. Homologous serum hepatitis. *Proc R Soc Med*. 1946;39(10):655-657.

[10]Paul JR. Infectious hepatitis. *Bull N Y Acad Med*. 1946;22:204-216.

[11]Neefe JR, Stokes J, Reinhold JG, et al. Hepatitis due to the injection of homologous blood products in human volunteers. *J Clin Invest*. 1944;23(5):836-855.

[12]Neefe JR, Gellis SS, Stokes J Jr. Homologous serum hepatitis and infectious (epidemic) hepatitis; studies in volunteers bearing on immunological and other characteristics of the etiological agents. *Am J Med*. 1946;1:3-22.

[13]Havens WP Jr. The etiology and epidemiology of infectious hepatitis. *Bull N Y Acad Med*. 1948;24(3):195-197; discussion 200.

[14]Krugman S, Giles JP, Hammond J. Hepatitis virus: effect of heat on the infectivity and antigenicity of the MS-1 and MS-2 strains. *J Infect Dis*. 1970;122(5):432-436.

[15]Feinstone SM, Kapikian AZ, Purceli RH. Hepatitis A: detection by immune electron microscopy of a viruslike antigen associated with acute illness. *Science*. 1973;182(116):1026-1028.

[16]Hilleman MR, Provost PJ, Miller WJ, et al. Development and utilization of complement-fixation and immune adherence tests for human hepatitis A virus and antibody. *Am J Med Sci*. 1975;270(1):93-98.

[17]Lemon SM, Binn LN. Serum neutralizing antibody response to hepatitis A virus. *J Infect Dis*. 1983;148(6):1033-1039.

[18]Armstrong ME, Giesa PA, Davide JP, et al. Development of the formalin-inactivated hepatitis A vaccine, VAQTA from the live attenuated virus strain CR326F. *J Hepatol*. 1993;18(Suppl 2):S20-S26.

[19]Lurman A. Eine Icterusepedemie. *Berlin Klin Wschr*. 1885;22:20-23.

[20]London WT, Sutnick AI, Blumberg BS. Australia antigen and acute viral hepatitis. *Ann Intern Med*. 1969;70(1):55-59.

[21]Dane DS, Cameron CH, Briggs M. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. *Lancet*. 1970;1(7649):695-698.

[22]Szmuness W, Stevens CE, Zang EA, et al. A controlled clinical trial of the efficacy of the hepatitis B vaccine (Heptavax B): a final report. *Hepatology*. 1981;1(5):377-385.

[23]Valenzuela P, Medina A, Rutter WJ, et al. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature*. 1982;298(5872):347-350.

[24]McAleer WJ, Buynak EB, Maigetter RZ, et al. Human hepatitis B vaccine from recombinant yeast. *Nature*. 1984;307(5947):178-180.

[25]Hilleman MR. Yeast recombinant hepatitis B vaccine. *Infection*. 1987;15(1):3-7.

[26]Shope RE. A transmissible tumor-like condition in rabbits. *J Exp Med*. 1932;56(6):793-802.

[27]Strauss MJ, Shaw EW, et al. Crystalline virus-like particles from skin papillomas characterized by intranuclear inclusion bodies. *Proc Soc Exp Biol Med*. 1949;72(1):46-50.

[28]Melnick JL, Bunting H, Banfield WG, et al. Electron microscopy of viruses of human papilloma, molluscum contagiosum, and vaccinia, including observations on the formation of virus within the cell. *Ann N Y Acad Sci*. 1952;54(6):1214-1225.

[29]Zur Hausen H. Human papillomaviruses and their possible role in squamous cell carcinomas. *Curr Top Microbiol Immunol*. 1977;78:1-30.

[30]Schiller JT, Lowy DR. Papillomavirus-like particle vaccines. *J Natl Cancer Inst Monogr*. 2001;(28):50-54.

[31]Hirst GK. Adsorption of influenza hemagglutinins and virus by red blood cells. *J Exp Med*. 1942;76(2):195-209.

[32]Smith W, Andrewes CH, Laidlaw PP. A virus obtained from influenza patients. *Lancet*. 1933;225:66.

[33]Francis T, Salk JE, Pearson HE, et al. Protective effect of vaccination against induced influenza A. *J Clin Invest*. 1945;24(4):536-546.

[34]Hare R, Curl M, McClelland L. The efficiency of the red cell adsorption and elution method for the preparation of influenza vaccine. *Can J Public Health*. 1946;37:284-291.

[35]Francis T Jr. Vaccination against influenza. *Bull World Health Organ*. 1953;8(5-6):725-741.

[36]Sabin AB. Epidemic encephalitis in military personnel; isolation of Japanese B virus on Okinawa in 1945, serologic diagnosis, clinical manifestations, epidemiologic aspects and use of mouse brain vaccine. *J Am Med Assoc*. 1947;133(5):281-293.

[37]Hoke CH, Nisalak A, Sangawhipa N, et al. Protection against Japanese encephalitis by inactivated vaccines. *N Engl J Med*. 1988;319(10):608-614.

[38]Sanchez JL, Hoke CH, McCown J, et al. Further experience with Japanese encephalitis vaccine. *Lancet*. 1990;335(8695):972-973.

[39]Tauber E, Kollaritsch H, Korinek M, et al. Safety and immunogenicity of a Vero-cell-derived, inactivated Japanese encephalitis vaccine: a non-inferiority, phase III, randomised controlled trial. *Lancet*. 2007;370(9602):1847-1853.

[40]Ra-zi-MiZ. *A Treatise on the Small-pox and Measles*. Trans. WA Greenhill. Sydenham Society; 1848.

[41]Panum P. *Observations Made During the Epidemic of Measles on the Faroe Islands in the Year 1846*. Bibliothek for Laeger; 1847.

[42]Enders JF, Peebles TC, McCarthy K, et al. Measles virus: a summary of experiments concerned with isolation, properties, and behavior. *Am J Public Health Nations Health*. 1957;47(3):275-282.

[43]Enders JF, Peebles TC. Propagation in tissue cultures of cytopathogenic agents from patients with measles. *Proc Soc Exp Biol Med*. 1954;86(2):277-286.

[44]Katz SL, Kempe CH, Black FL, et al. Studies on an attenuated measles-virus vaccine. VIII. General summary and evaluation of the results of vaccine. *N Engl J Med*. 1960;263:180-184.

[45]Stokes J Jr, Weibel RE, Villarejos VM, et al. Trivalent combined measles-mumps-rubella vaccine. Findings in clinical-laboratory studies. *JAMA*. 1971;218(1):57-61.

[46]Strebel PM, Dayan GH, Halsey NA. Measles vaccine. In: Orenstein PA, ed. *Vaccines*. Saunders; 2004.

[47]Shinefield H, Black S, Digilio L, et al. Evaluation of a quadrivalent measles, mumps, rubella and varicella vaccine in healthy children. *Pediatr Infect Dis J*. 2005;24(8):665-669.

[48]Johnson CD, Goodpasture EW. An investigation of the etiology of mumps. *J Exp Med*. 1934;59(1):1-19.

[49]Johnson CD, Goodpasture EW. The histopathology of experimental mumps in the monkey, macacus rhesus. *Am J Pathol*. 1936;12(4):495-510.7.

[50]Hilleman MR, Buynak EB, Weibel RE, et al. Live, attenuated mumps-virus vaccine. *N Engl J Med*. 1968;278(5):227-232.

[51]Underwood M. *Debility of the Lower Extremities in a Treatise on the Diseases of Children*. J Matthews; 1789.

[52]Enders JF, Weller TH, Robbins FC. Cultivation of the Lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. *Science*. 1949;109(2822):85-87.

[53]Robbins FC, Enders JF, Weller TH. The effect of poliomyelitis virus upon cells in tissue cultures. *J Clin Invest*. 1950;29(6):841.

[54]Brodie M. Active immunization against poliomyelitis. *Am J Public Health Nations Health*. 1935;25(1):54-67.

[55]Kolmer JA. Vaccination against acute anterior poliomyelitis. *Am J Public Health Nations Health*. 1936;26(2):126-135.

[56]Salk JE. Immunization against poliomyelitis. *Pediatr Clin North Am*. 1953;1(1A):49-51.

[57]Salk JE. Vaccination against paralytic poliomyelitis performance and prospects. *Am J Public Health Nations Health*. 1955;45(5 Pt 1):575-596.

[58]Sabin AB. Oral, live poliovirus vaccine for elimination of poliomyelitis. *Arch Intern Med*. 1960;106:5-9.

[59]Sabin AB. Eradication of poliomyelitis—present status and future prospects. *R I Med J*. 1962;45:453-462.

[60]Nathanson N, Langmuir AD. The Cutter incident. Poliomyelitis following formaldehyde- inactivated poliovirus vaccination in the United States during the spring of 1955. II. Relationship of poliomyelitis to Cutter vaccine. *Am J Hyg*. 1963;78:29-60.

[61]Horsley V. On rabies: its treatment by M. Pasteur, and on the means of detecting it in suspected cases. *Br Med J*. 1889;1(1468):342-344.

[62]Roux M. The Croonian lecture on preventive inoculation. *Br Med J*. 1889;1(1484):1269-1274.

[63]Lackenbach FI. Rabies and the Pasteur treatment. *Cal State J Med*. 1912;10(3):123-125.

[64]Bishop RF, Davidson GP, Holmes IH, et al. Detection of a new virus by electron microscopy of faecal extracts from children with acute gastroenteritis. *Lancet*. 1974;1(7849):149-151.

[65]Wyatt RG, James WD, Bohl EH, et al. Human rotavirus type 2: cultivation in vitro. *Science*. 1980;207(4427):189-191.

[66]Beards GM, Pilfold JN, Thouless ME, et al. Rotavirus serotypes by serum neutralisation. *J Med Virol*. 1980;5(3):231-237.

[67]Gregg NM. Congenital cataract following German measles in the mother. 1941. *Aust N Z J Ophthalmol*. 1991;19(4):267-276.

[68]Neva FA, Weller TH. Rubella interferon and factors influencing the indirect neutralization test for rubella antibody. *J Immunol*. 1964;93:466-473.

[69]Parkman PD, Buescher EL, Artenstein MS. Recovery of rubella virus from army recruits. *Proc Soc Exp Biol Med*. 1962;111:225-230.

[70]Meyer HM Jr, Parkman PD, Panos TC. Attenuated rubella virus. II. Production of an experimental live-virus vaccine and clinical trial. *N Engl J Med*. 1966;275(11):575-580.

[71]Meyer HM Jr, Parkman PD, Hobbins TE, et al. Clinical studies with experimental live rubella virus vaccine (strain HPV-77). Evaluation of vaccine-induced immunity. *Am J Dis Child*. 1968;115(6):648-654.

[72]Reef SE, Cochi SL. The evidence for the elimination of rubella and congenital rubella syndrome in the United States: a public health achievement. *Clin Infect Dis*. 2006;43(Suppl 3):S123-S125.

[73]Plotkin SA. The history of rubella and rubella vaccination leading to elimination. *Clin Infect Dis*. 2006;43(Suppl 3):S164-S168.

[74]Pattanayak S, Arora DD, Sehgal CL, et al. Comparative studies of smallpox vaccination by the bifurcated needle and rotary lancet techniques. *Bull World Health Organ*. 1970;42(2):305-310.

[75]Rubin BA. A note on the development of the bifurcated needle for smallpox vaccination. *WHO Chron*. 1980;34(5):180-181.

[76]Breman JG, Arita I. The confirmation and maintenance of smallpox eradication. *N Engl J Med*. 1980;303(22):1263-1273.

[77]Takahashi M, Okuno Y, Otsuka T, et al. Development of a live attenuated varicella vaccine. *Biken J*. 1975;18(1):25-33.

[78]Centers for Disease Control and Prevention. Licensure of varicella virus vaccine, live. *MMWR Morb Mortal Wkly Rep*. 1995;44(13):264.

[79]Weller TH. Varicella: historical perspective and clinical overview. *J Infect Dis*. 1996;174(Suppl 3):S306-S309.

[80]Reed W, Carroll J, Agramonte A, et al. The etiology of yellow fever—a preliminary note. *Public Health Pap Rep*. 1900;26:37-53.

[81]Reed W. Recent researches concerning the etiology, propagation, and prevention of yellow fever, by the United States Army Commission. *J Hyg (Lond)*. 1902;2(2):101-119.

[82]Finlay CJ. New aspects of yellow fever etiology arising from experimental findings of the last three years. *Public Health Pap Rep*. 1903;29:68-70.

[83]Theiler M. Susceptibility of white mice to the virus of yellow fever. *Science*. 1930;71(1840):367.

[84]Theiler M, Smith HH. The use of yellow fever virus modified by in vitro cultivation for human immunization. *J Exp Med*. 1937;65(6):787-800.

FDA, U.S. Food and Drug Administration; HBsAg, hepatitis B surface antigen; HPV, human papillomavirus; VLP, virus-like particle; ID, intradermal; JEV, juvenile encephalitis virus; IPV, inactivated poliovirus vaccine; WHO, World Health Organization.

Origins of Vaccinology

Origin of Live Virus Vaccination

Most viral vaccines were developed because of both fortuitous events and empirical findings. The origin of modern vaccinology, and particularly live virus vaccination, is based on the practice of variolization (mechanical attenuation and intentional low-dose infection) to reduce the virulence of subsequent smallpox infection, which was initiated more than 1000 years ago in India and China. From this practice arose the concept of vaccination (using an alternative agent to provide immunity against an infectious disease), and the first vaccine used in humans, live cowpox virus. In 1796, Edward Jenner inoculated 8-year-old James Phipps with purulent material taken from a cowpox pustule on the hand of milkmaid Sarah Nelmes and introduced it into an incision on the boy's arm. Jenner subsequently proved that the boy was protected from an inoculation of material from a smallpox lesion. He published the work in 1798 and coined the word vaccine from the Latin *vacca* for cow and designated the process vaccination. For 80 years, live virus was transferred almost exclusively from human arm to arm by collection of purulent material. Subsequently, vaccine was propagated on the skin of calves using serial inoculation resulting in vaccinia virus as the distinct vaccine strain.[277] This vaccine was used successfully to eradicate smallpox from the population, and smallpox remains the only human viral infection to be validated as eradicated through a vaccination campaign (Fig. 15.1). A concerted effort to eradicate polioviruses is ongoing.

FIGURE 15.1 **Origins of live attenuated viral vaccines**. **A**. Edward Jenner is credited for performing the first vaccine experiment in which he demonstrated that inoculation of a boy with purulent material obtained from a cowpox lesion on the hand of a milkmaid protected him against subsequent challenge with variola. This event launched the formal concept of vaccination and established the prototypical approach for developing live attenuated viral vaccines. **B**. The implementation of smallpox vaccination faced many of the same issues of fear and misunderstanding that are prevalent today and impede the deployment of effective vaccines as illustrated by a political cartoon published in 1804. **C**. The successful eradication of smallpox yielded many lessons about the value of simple, low-cost, local production of vaccine as illustrated by the collection of vaccine material from the hide of a cow during the initial phases of the WHO-sponsored eradication campaign. There were also lessons on the importance of product stability and simple delivery approaches. **D**. Once vaccinia was lyophilized, it could be reconstituted on site and was not as dependent on maintenance of the cold chain. The bifurcated needle **(D)** was an innovation that provided a simple standardized approach to inoculation without the need for needles or mechanical devices and allowed vaccination to penetrate remote locations. (**A and B**, courtesy of the National Library of Medicine; **C**, courtesy of J. Mohr, WHO; **D**, courtesy of James Gathany/CDC.)

Origins of Inactivated Virus Vaccination

In the 1880s, Louis Pasteur and his colleagues attributed rabies to virus replication in the central nervous system. Next, they discovered that drying rabies-infected tissue at room temperature attenuated the infectivity of the tissue. The dried rabies-infected tissue was determined to be noninfectious, yet serial inoculation of dogs with the tissues provided protection against an otherwise lethal rabies virus challenge. Inoculation of dried rabies-infected central nervous system tissue was tested in a single human postexposure case in 1885 when Pasteur serially inoculated Joseph Meister, a 9-year-old boy bitten by a rabid dog more than 2 days earlier. The initial inoculations contained highly attenuated virus from rabbit spinal cord dried for 15 days, followed by inoculations with less attenuated virus-infected tissue. In total, the regimen included 13 immunizations over 10 days. The boy was fully protected from disease, and this intervention represents the beginning of an era in which inactivated vaccines were used for other viral diseases following Pasteur's paradigm of identification, inactivation, and inoculation (Fig. 15.2; e-Table 15.1).

FIGURE 15.2 **Origins of inactivated viral vaccines**. Louis Pasteur is pictured here as the person responsible for the inactivated viral vaccine concept. His work on the rabies vaccine produced by desiccation of spinal cords from rabid dogs also anticipated the public debates that still occur involving the balance of risk and benefit to the individual and the public from the implementation of vaccines. (Courtesy of the National Library of Medicine.)

Critical Events in the History of Viral Vaccines

Since the introduction of vaccinia virus, licensed vaccines have been developed for nearly 30 virus strains from 16 types of viral pathogens (Table 15.1). Notable advances in viral vaccine development over the past century include semieradication of many viral infections, processes for optimization of manufacturing cell lines for next-generation vaccines, utilization of human immune correlates to establish efficacy for licensure, and improvements in vaccine safety monitoring, which is important for public safety but also serves to keep safe and efficacious vaccines from unwarranted negative assessment. The COVID-19 pandemic saw a major increase in the rapidity of vaccine development. Prior to 2020, the 4-year mumps vaccine development process was the fastest ever from obtaining a virus sample to approval of a vaccine. In 2020, the first SARS-CoV-2 virus genome sequences were reported on January 10 and by December 2 (<11 months later) the Pfizer/BioNTech COVID-19 mRNA vaccine was approved for emergency use in the United Kingdom after being fully tested in clinical trials.

TABLE 15.1 Viral vaccines licensed in the United States[a]

[a]See individual chapters for specifics on target populations and immunization schedules for individual agents, which may vary or change from this overview. In addition, see the following website for a list of licensed vaccine products, including combination products: http://www.fda.gov/cber/vaccine/licvacc.htm.

[b]RotaShield tetravalent vaccine is no longer available. RotaTeq pentavalent and Rotarix monovalent vaccines are available.

[c]Zoster vaccine live (Zostavax) is no longer available for use in the United States, as of November 18, 2020.

VLP, virus-like particle; WT, wild-type; IM, intramuscular; SQ, subcutaneous; ID, intradermal.

Goals of Immunization Against Viral Diseases

Prevention of Disease or Infection

The major goals of immunization against viruses are the prevention or modification of disease in an individual and the control of epidemic infection within populations. Most viral vaccines prevent or modify disease without necessarily preventing infection. The ways in which this objective is achieved are diverse. Whereas most viral vaccines are administered before infection occurs, prevention of CNS disease in individuals inoculated with rabies virus by an animal bite can be achieved by administration of a vaccine regimen initiated shortly after infection has occurred. This *postexposure prophylaxis* is possible because the long incubation period of the infection permits the development of an effective immune response in time to modify infection and prevent disease. Similarly, infants born to untreated hepatitis B–infected mothers are treated with postexposure vaccination and antibody treatment. Interestingly, even for viruses with a more rapid course, like variola, individuals can be protected against a fatal outcome if vaccinia virus vaccine is administered up to 4 days after the initial exposure to the virus. This effect may be due to augmented innate immune responses or more rapid induction of adaptive immune responses.

The major goal of immunization against rubella virus is prevention of fetal abnormalities caused by intrauterine infection. To achieve this goal, both males and females are immunized in the United States. Immunization of males has little direct benefit to the vaccinee other than prevention of the relatively mild illness caused by the rubella virus, and males are not targeted in all national vaccine programs. The fetus of a vaccinated female is protected by immunization of the mother before pregnancy. Community (herd) immunity resulting from extensive immunization of males and females provides some protection to the fetus of an unvaccinated female by decreasing the circulation of virus in the community. An example of community immunity was seen early after introducing the mumps vaccine. The incidence of mumps declined precipitously before vaccine coverage was significantly expanded. A modest reduction in the number of susceptible persons and transmitters had a significant impact on the epidemic.91a

In addition to reducing the frequency of susceptible persons and transmitters, some live viral vaccines can improve community immunity by the spread of vaccine from recipients to contacts. This phenomenon has been observed with oral live poliovirus vaccine[406] resulting in the indirect immunization of a significant proportion of the population and a marked reduction in the incidence of poliomyelitis in both vaccinated and "unvaccinated" individuals. Spread of vaccine virus has been viewed by many as a desirable property because this transmission confers the benefits of immunization on many individuals who were not vaccinated. However, spread of vaccine in the population also raises concern because of the rare occurrence of vaccine-associated paralytic disease among contacts of vaccinees, and immunization of persons without their prior consent presents ethical issues. For instance, wild poliovirus type 2 transmission stopped in Africa in the mid-1990s, but hundreds of cases of circulating vaccine-derived poliovirus type 2 (cVDPV2) were reported in Nigeria in 2021 during the COVID-19 pandemic, and cases are still occurring in 2022.

Although most vaccines in use today are effective in preventing disease, they usually are inefficient at completely preventing infection. Prevention of infection is a desirable property for specific vaccines such as HBV and HIV. The vaccine for human papillomavirus (HPV) was licensed based on its ability to prevent cervical neoplasia.[382] In addition to preventing the acute effects of viral infection, the HBV vaccine is also given with the goal of preventing hepatomas.[212] Infection with HBV during the neonatal period is associated with an increased risk of progression to the chronic carrier state and associated with the development of chronic liver disease and hepatocellular carcinoma later in life. Therefore, a major goal of HBV vaccination is to prevent transmission of infection from persistently infected mothers to their infants during the neonatal period. Considerable success in this effort has been achieved using HB surface antigen (sAg) vaccine or hyperimmune HBV antiserum plus vaccine immediately after delivery.[212] HIV is a lentivirus, and the possibility of progression to AIDS can occur many years after initial infection. Therefore, it is desirable to develop a vaccine that induces immunity to prevent infection or result in an abortive or transient HIV infection. We have not successfully created a vaccine for HIV, however, because of the extreme genetic diversity of HIV genome sequences, the difficulty of eradicating infected cells in which virus genome has integrated into host DNA but is not replicating, and other factors. We also have failed to develop licensed vaccines for respiratory syncytial virus (RSV), because the target populations of infants and the elderly mount less effective immune responses to immunizations, mucosal immunity is hard to maintain at high levels for prolonged periods, and additional barriers.

Immunotherapy

HSV-1 or HSV-2 as well as VZV produce latent infections in sensory ganglia that periodically reactivate, resulting in development of new lesions and increased opportunity for transmission of virus. Therefore, a goal of immunization of persons previously infected with these viruses is to prevent or ameliorate recurrent disease caused by reactivation of their latent infection.[38] This form of immunization is referred to as immunotherapy. Other illnesses that are candidates for immunotherapeutic vaccines include warts and cancer caused by HPV and chronic hepatitis caused by HBV or HCV. HIV-1 has also been considered as a target for immunotherapy to prevent the long-term consequences of chronic infection or to reduce the requirements for ongoing antiretroviral drug therapy. Because of the extent of repeated Ag exposure and damage to many elements of the adaptive immune system resulting in immunodeficiency, it may be difficult to elicit new immune responses to effectively control virus infection and the associated disease.[67] Effective immunotherapeutic vaccines for recurrent HSV face a similar challenge since the vaccinees previously infected by wild-type virus are repeatedly reimmunized during each recurrence. However, for a related herpesvirus, VZV, where recurrence is relatively uncommon, immunization with live attenuated vaccine reduces the incidence of herpes zoster and postherpetic neuralgia.[108]

Eradication or Elimination

Appropriate use of a highly effective vaccine can help to eradicate a major disease when humans are the only natural host for the virus, and there is no natural reservoir or intermediate host. During the 1970s, this goal of eradication was achieved for smallpox largely using live vaccinia virus vaccine. This campaign ranks as one of the major public health achievements in all history. Two other human viruses have been targeted similarly for eradication, namely poliovirus and measles virus, and significant progress toward these goals has been made for both viral pathogens. The United Nations officially declared eradication of the morbillivirus animal pathogen rinderpest in 2011, after a concerted worldwide eradication program. Unfortunately, eradication of viral pathogens usually is an elusive goal because of setbacks from social instability, antivaccine sentiments, the threat of bioterrorism, and the potential for intentional reintroduction or resurrection of previously eradicated viral pathogens. Therefore, it will be prudent to find ways of maintaining immunity to serious virus pathogens even after eradication of virus in the natural reservoir has been achieved. The new technologies that have improved our ability to identify and perform research on emerging pathogens have also created the capacity to synthetically recreate pathogens (ie, to "rescue" infectious viruses from synthetic DNA copies of virus genomes). Elimination of a virus pathogen suggests that epidemic and endemic disease is controlled, and no active cases are present. However, setting the goal of elimination acknowledges the possibility of reemergence and may include maintenance of active vaccine-induced immunity. For instance, wild poliovirus type 2 was declared eradicated by the Global Commission for the Certification of Eradication of Poliomyelitis (GCC) in September 2015, and in May 2016, the world stopped using oral polio vaccine type 2 (OPV2) for routine vaccination of children. Many specimens stored by researchers however, such as stool or respiratory secretion specimens collected at times and in geographic areas where wild poliovirus type 2, vaccine-derived poliovirus type 2, oral polio vaccine type 2, or Sabin type 2 viruses were circulating, are likely to contain these viruses. The World Health Organization (WHO) Global Action Plan (GAPIII) for eradication of poliovirus states that each country is required to complete a national inventory of poliovirus-containing materials. Thus, authorities currently are surveying nonpoliovirus facilities to help them identify specimens potentially infected with poliovirus types 1, 2, or 3 and to implement appropriate measures for their destruction or safe and secure handling and storage.

Semieradication Secondary to Effective Vaccine Campaigns

Several vaccines have resulted in regional semieradication of the virus disease or dramatic reduction in the incidence or morbidity from infection (polio, measles, mumps, rubella, rabies, and VZV). Other vaccines are highly effective and have been reduced to use only for travelers to endemic areas of the world (yellow fever, Japanese encephalitis). Licensed vaccines for hepatitis A and B are highly effective and for influenza are moderately effective, and their impact on frequency of infection has been improved by new strategies for distribution (Table 15.2).

TABLE 15.2 Impact of licensed vaccines on viral diseases with well-defined surveillance and estimated impact on disease burden for other viral vaccines

[a]http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5953a1.htm

[b]http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5933a1.htm

[c]http://www.cdc.gov/rabies/location/usa/surveillance/index.html

[d]http://www.merck.com/product/usa/pi_circulars/v/varivax/varivax_pi.pdf

[e]Last U.S. epidemic in New Orleans.

How Vaccines Work

The general strategy of vaccination is to cause an antigenic exposure that faithfully mimics exposure to the antigens of a pathogen to induce protection against infection or disease on subsequent exposure, while causing the least side effects possible. Virus vaccines can be live attenuated (weakened) strains that cause infection but not disease, or they can be created by incorporation of inactive components of the virus (antigens) or the genes encoding such antigens. The key to efficacy of a vaccine is induction of robust and durable vaccine-induced immunity.

Correlates of Protection

The goal of studies of correlates of protection (CoPs) is to define the immune responses that are responsible for the efficacy of a vaccine. CoPs enable the selection of the most appropriate and protective antigens and the monitoring of a parameter that tracks with protection in individuals or populations after vaccination. If an efficacy trial in not feasible (or a simple vaccine strain change is warranted) CoPs help manufacturers to obtain licensure, measure lot-to-lot consistency of vaccines, and bridge from one formulation or construct to another. The eminent vaccinologist Stanley Plotkin has written summaries of CoPs that shape the field by simplifying the concepts,[372] distinguishing biological from statistical CoPs,[375] and pointing out that CoPs are complex because the immune system is complex and often exhibits redundant capacity to respond to infection.[373] Plotkin defined three principal terms relating to CoPs: A *CoP* is a marker of immune function that statistically correlates with protection after vaccination, which may be mechanistic (*mCoP*) or nonmechanistic (*nCo*P). The nCoP is defined as an immune factor that does not cause protection but nevertheless predicts protection through its (partial) correlation with another immune response(s) that mechanistically protects. Diverse immune factors have been noted as CoPs for vaccines (e-Fig. 15.3), including (1) antibodies (binding, infection neutralizing, mucosal IgA or IgG, antibody-dependent cellular cytotoxicity (NK cells), antibody-dependent cellular phagocytosis, histoblood group antigen blocking); (2) CD4+ T cells (cytokine production [IFN-γ, IL-2, TNF-α], help to antibody production, effector memory, central memory); and (3) CD8+ T cells (cytotoxicity, effector memory, central memory, Th17+ T-cell secretion of IL-17 on mucosa).[374] Traditionally, the assessment of vaccine efficacy has been made by determining whether infection or disease has been prevented. In the process of establishing vaccine efficacy, the use of a human immune CoP is potentially more efficient and has been implemented successfully in the yearly approval process for influenza vaccines. Influenza vaccines typically are approved based on the accepted surrogate of protection, a hemagglutination inhibition (HAI) titer ≥1:40 or a fourfold increase over the prevaccination HAI titer. In this case, the immune response serves as a surrogate marker that is predetermined to predict clinical benefit, and therefore, the lengthier process of assessing the presence or absence of influenza infection in a clinical trial is not always needed.[462] Similarly, assessing the result of infection (or absence of), rather documenting an effect on the virus itself can also be used to assess protective efficacy. The HPV vaccines were licensed based on efficacy derived from the prevention of cervical and

vulvar cancer, precancerous lesions, and dysplastic lesions.[461]

e-FIGURE 15.3 **Relative importance of vaccine-induced immune responses to different stages of viral life cycle.** Ab is the only component of the adaptive immune response that provides a defense against a cell-free virion. In contrast, T cells can only recognize virus-infected cells because of the requirement for host cell MHC-restricted Ag presentation. Ab has some mechanisms involving the Fc portion that can bind complement or Fc receptors on NK cells to mediate killing of virus-infected cells. If a virus has the capacity for latency (eg, HSV or HIV), or sequestration (eg, HIV harbored in extracellular space of lymph nodes bound to follicular dendritic cells), or infection of immunoprivileged site (eg, brain or eye), it can remain hidden or protected from immune responses. Antibody Fc regions also interact with other cells to mediate diverse activities, including antibody-dependent cellular phagocytosis (ADCP) mediated by monocytes, antibody-dependent neutrophil-mediated phagocytosis (ADNP), antibody-dependent cell-mediated viral inhibition (ADCVI), and antibody-dependent complement-mediated lysis (ADCML) of cells or virions.

Mechanisms of Vaccine-Induced Immunity

Immune mechanisms have evolved to protect the host against a diverse assortment of viral pathogens. Viruses and their hosts co-evolve; viruses find ways to evade host defenses and hosts adapt mechanisms to control virus replication. The host is equipped with three principal toolsets to resist infection: (1) physical or mechanical barriers (such as skin or gut, genital, or respiratory tract mucosa), (2) innate immune mechanisms that can limit the initial encounter with a virus, and (3) adaptive immune mechanisms that provide immunity against repeated exposures to the same viral pathogen. Although innate responses are important during the induction phase of vaccine-induced immunity, humoral and cellular adaptive responses are the primary effector mechanisms of protection from future infection (e-Fig. 15.4). Humoral and cellular responses are complementary in that Ab prevents infection by cell-free virus, but when infection occurs despite innate defenses and the presence of neutralizing Abs, T-cell responses and antibodies that bridge infected cells and Fcγ receptor–bearing immune cells are designed to eliminate virus-infected cells and diminish the subsequent release of infectious virus. The repertoires of B- and T-cell receptors have also evolved complementary strategies to deal with genetic variation in viruses. For a given antigenic site on a viral protein, most hosts can expand a B-cell population that can recognize the structure through clonal lineage development of variant antibodies using somatic hypermutation (SHM). This mechanism may lead to population-wide protection against reinfection with a particular virus or minimally drifted virus variants. This immune pressure may cause selection of escape viruses bearing major antigenic changes, sometimes resulting in a new clade, subgroup, group, subtype, or serotype, which is not recognized by the original antibodies. Therefore, the host must start over in the generation of antibody-mediated type-specific protection. In contrast, T-cell epitopes are not shared between hosts unless they happen to share the same MHC-restricting allele. Consequently, each infected host generates T-cell responses against a limited number of viral epitopes that are largely determined by HLA haplotype. Unlike antibody epitopes, T-cell epitopes are often cross-reactive between viral serotypes and thereby provide the antigen-experienced host with partial protection against new virus strains until the antibody responses can adapt.

e-FIGURE 15.4 **Virus infection engages the major components of the adaptive immune response. A.** The principal components of the adaptive immune system are B cells and T cells. The surface structures of virions are accessible to antibodies (ie, B-cell receptors) that are important for virus recognition and neutralization. Production of virus proteins in the cytoplasm leads to processing of virus-derived peptides in the proteasome and transport to the endoplasmic reticulum where they can associate with MHC class I molecules for display on the cell surface and recognition by CD8 T cells. Virus particles and proteins that are endocytosed can associate with MHC class II proteins in lysosomes for presentation to CD4 T cells. **B.** Timing is everything. In a prototypical viral infection, the virus penetrates physical and innate immune barriers to infection to establish a local infection that progresses to infect the target organ of disease. This can occur through local or systemic spread. Natural immunity occurs after these acute events and in most cases successfully clears or controls infection to limit disease. In some cases, virus infection can become persistent. **C.** The virological and clinical consequences of viral infection and the host immune response are shown for persistent viral infections like HIV or for a self-limited viral infection like influenza or dengue. Note that the clinical manifestations of a persistent or cytolytic virus infection are congruent with the viral load, while in self-limited infections, the peak illness can occur at the time of peak viremia or may be temporally associated with the timing of innate or adaptive immune responses.

When a virus breaches the physical and innate barriers and infects a target cell, several rounds of replication may occur in a primary infection before the adaptive immune responses are established (e-Fig. 15.4B). Depending on the virus, this exposure may result in spread to the target organ of disease and/or establishment of a persistent infection. The timing, magnitude, location, and qualitative features of the host response determine the clinical outcome of infection. In persistent virus infections (eg, HIV, HCV, or HSV), disease manifestations are often congruent with virus replication. In self-limited infections (eg, those caused by influenza, RSV, or dengue), the bulk of disease often occurs after the peak of viral replication, although in some cases, disease is worst near the peak in viral load (e-Fig. 15.4B). In cases of persistent or latent virus infection, the immune response attempts to limit virus replication or spread. Control of the magnitude and composition of complex immune responses is critical to avoid immunopathology. Fortunately, there is considerable redundancy inherent in immunity to viruses (e-Fig. 15.4C). For example, while CD8+ T cells are the primary mediator of cytolytic activity, this function may also be accomplished by complement or by NK cells targeting the Fc portion of Ab labeling virus-infected cells. Another well-studied example of this redundancy involves CD4 and CD8+ T cells, either of which are sufficient to achieve clearance of virus, as shown in studies of influenza A virus or poxviruses in mice. Animals lacking CD8+ T cells readily clear influenza virus or vaccinia virus from the lungs, indicating that CD4 T cells and Ab are sometimes sufficient for viral clearance.[289,418] The therapeutic efficacy of Abs acting in the absence of other immune functions has also been demonstrated in experimental influenza A virus infection of mice. Abs to the influenza A virus surface HA cleared virus from the lungs of persistently infected SCID mice.[418] While CD8+ T cells are specialized in recognition and clearance of virus-infected cells, CD4 T cells (plus the Abs secreted by B cells for which they provide help) and CD8+ T cells can mediate viral clearance independently. The relative importance of these two arms of the immune response varies in the resolution of viral infections. Even in the case of lymphocytic choriomeningitis virus (LCMV), the prototypic arenavirus, which in the murine model is a paradigm for CD8+ T-cell–mediated immunity, Abs and CD4 T cells each make a significant contribution to the resolution of primary viral infection.[19] In general, Abs are the major mediator of resistance to reinfection with virus, and CD8+ T cells are the major mediator of clearing virus-infected cells. CD4 T cells are critical for the induction of robust Ab responses and important for “helping” memory CD8+ T-cell responses and have potential for direct antiviral effector functions. Abs work best when the target organ for disease pathogenesis is distinct from the initial site of infection, particularly in cases where the virus must travel through the bloodstream to reach its target organ. For example, in diseases like measles, polio, hepatitis, or most viral encephalitides, where viremia must be established to cause disease, it often takes very small concentrations of preexisting Abs to achieve clinical protection. Since it is rarely possible to elicit sufficient Ab through vaccination to prevent virus infection completely, other immune mechanisms must contribute to vaccine-induced immunity.

One reason that live virus vaccines are so effective is that many components of both innate and adaptive immunity are engaged. The protein,

nucleic acid, carbohydrate, and lipid components of viruses induce innate responses through a variety of Toll-like receptor (TLR) and other pathogen-associated molecular pattern (PAMP) receptor interactions. Virus infection induces all components of the adaptive immune response—Ab responses are induced against viral surface proteins in their native conformation, CD4 T cells are stimulated, and viral proteins are produced in the cytoplasm, processed, and presented in MHC class I molecules for induction of CD8+ CTL responses. All these responses contribute to the clinical outcome from a virus infection. Abs and innate defenses provide the initial barrier to infection by neutralizing incoming virus. After the virus has infected a cell, however, it is then incumbent on the innate defenses to control the virus until the adaptive cellular immune response can be activated, expand, and traffic to the affected tissues. In the immune host, preexisting antibody prevents or limits the number of infected cells, and rapid mobilization of memory T-cell responses rapidly clears virus-infected cells. While cytolytic T cells are potent in their ability to clear virus-infected cells and reduce the spread of virus, this activity often comes at a cost. The T-cell response can injure the host as it clears the virus and is thus tightly regulated. Turning the immune response off as virus infection resolves is just as important as turning it on for the well-being of the host. The system is biased toward controlling inflammation and that may be a reason why the T-cell response is regulated to require several days to activate and expand. Influencing the events in this period between virus exposure and the fully activated primary T-cell response is one of the key factors in determining the efficacy of vaccine-induced immunity, for which the goal is to prevent, abort, or control viral infection to avoid clinical disease. The cytokine environment in which T cells are induced stimulates diverse patterns of differentiation (e-Fig. 15.5).

e-FIGURE 15.5 **Vaccine-induce cytokine milieu influences patterns of T-cell differentiation.** The primary effectors of adaptive immunity are regulated by earlier events in the immune response. The virus-infected cell produces chemokine gradients to recruit the cells needed for the initial wave of innate immune responses and Ag processing by professional Ag-presenting cells (APCs). These cells also produce cytokines and engage in cognate interactions to influence the function of cells involved in the inflammatory response. APCs also can produce a variety of cytokines and cognate interactions to initiate the adaptive immune response. This is largely orchestrated by CD4+ T cells that have been characterized phenotypically and functionally distinct subpopulations. The pattern of CD4 T-cell response elicited by the initial Ag exposure to vaccination or infection has a significant influence on functional properties of the adaptive immune response on subsequent exposure to Ag.[174]

The aggregate data suggest that a successful strategy of immunoprophylaxis against most virus infections requires the generation of an immune response to the surface Ags displayed on virions and on virus-infected cells. Other viruses (such as poxviruses, herpesviruses, filoviruses, some respiratory viruses, and lentiviruses) that have a more complex pathogenesis may require vaccines that induce more complex immune responses for protection from infection or control of disease. Many of these viruses possess the capacity for persistence, infection of immunoprivileged sites, use sophisticated immune evasion strategies, directly infect their target organ without a requirement for viremia, or cause disease by eliciting an aberrant host response. Vaccine development for these viruses probably will require the induction of neutralizing Abs against surface proteins to protect against infection in addition to T-cell responses against structural and regulatory proteins to control virus spread and to modulate the composition of the immune response.[178]

Vaccine-Induced Humoral Immunity

The correlates of immunity that have been established to date for licensed viral vaccines have been associated with Ab responses. In fact, the rationale for development of many of the vaccines in use today was the evidence derived from clinical trials of Ab treatments showing that Abs could mediate protection against disease. Passive transfer studies established the efficacy of Abs in preventing or treating viral infections and disease caused by a wide variety of viruses belonging to diverse RNA or DNA virus families that include the orthomyxoviruses, paramyxoviruses, alphaviruses, flaviviruses, arenaviruses, lentiviruses, picornaviruses, hepadnaviruses, and herpesviruses. Examples of the licensed clinical use of immune globulins include preparations for hepatitis A and B viruses, measles virus, varicella-zoster virus, rabies virus, RSV, and cytomegalovirus (CMV). Many new human monoclonal antibodies (mAbs) have been discovered in the last decade, and some have been licensed or authorized for prevention or treatment of RSV, Ebola virus, HIV, and SARS-CoV-2.

A long history of Ab treatments laid the groundwork for current vaccine strategies. Commercial human gamma globulin, which usually contains 16%-18% immunoglobulin G (IgG), is highly effective in preventing hepatitis A disease and was used widely for that purpose during the past 50 years, even though only a small proportion of the Abs in the preparations are specific for that virus.[97] Currently, inactivated vaccines for hepatitis A are licensed and thought to work by inducing serum Abs to the virus. An historic double-blind, prospective clinical trial performed in 1951-1952 showed that human IgG also was effective in preventing paralytic disease caused by poliovirus.[196] Intramuscular inoculation of 0.3 mL of IgG/kg conferred significant resistance that lasted for at least 5 weeks. This seminal observation showed that Abs alone can confer resistance to poliomyelitis and suggested that vaccines that induced such Abs might protect against the disease. Subsequent field trials of inactivated vaccines by Salk and live attenuated vaccines by Sabin validated this view, leading to implementation of universal immunization against poliovirus and ultimately the current worldwide campaign for elimination of polio disease (Fig. 15.3). Before the live measles virus vaccine was licensed for routine use in immunization of young children, human IgG was used for passive prophylaxis and was the mainstay for prevention of disease. IgG given at a dose of 0.5 mL/kg prevented measles, even when administered to exposed individuals as late as 3 days after inoculation with virus.[219] Human IgG prepared from pooled plasma selected for a high titer of varicella-zoster virus Abs (varicella-zoster immune globulin or VZIG) is licensed in the United States for use in prevention of severe varicella in immunosuppressed children.[507] Most of the exposed immunosuppressed children receiving treatment still become infected, but the majority exhibit attenuated disease or subclinical infection. Live attenuated varicella virus vaccine was licensed and recommended for universal immunization in 1995. Human IgG was effective in preventing chronic hepatitis B virus (HBV) infection in high-risk infants following maternal exposure.[31] Hepatitis B immune globulin and subunit vaccine are now used in concert to protect infants in this setting.

FIGURE 15.3 **Polio vaccine development**. The development of vaccines for polio brought the process of vaccine development into the public eye and was the first licensed vaccine that relied on mammalian cell culture. Jonas Salk **(A)** spearheaded the development of the inactivated polio vaccine (IPV) **(B)**. The development of the oral polio vaccine (OPV) **(C)** was headed by Albert Sabin **(D)**. These two vaccines are still in use today, and over the last 50 years have nearly eliminated poliovirus making the goal of global eradication feasible. (**A**, ©AP Wide World Photos; **C courtesy of Division of Medicine and Science, National Museum of American History, Smithsonian Institution. D**, courtesy of the Hauck Center for the Albert B. Sabin Archives, Henry R. Winkler Center for the History of the Health Professions, University of Cincinnati Libraries.)

Preclinical studies in animals suggested that serum RSV neutralizing Abs above a threshold titer were protective against lower respiratory tract infection, and this observation was validated during a clinical trial of human IgG containing a high titer of RSV neutralizing Abs that led to licensure in 1995.[379,387] Of interest, the level of serum RSV neutralizing Abs required for reduction of hospitalization was like that needed to

prevent infection in cotton rats. A second-generation approach, the use of a humanized murine neutralizing mAb to RSV, palivizumab, was licensed in 1998.[349] Palivizumab was the only mAb licensed for prevention of a viral disease for many decades. These clinical applications strongly suggest that neutralizing Abs is an important correlate of protection for RSV vaccines, which are under development. A second-generation long-acting RSV mAb (nirsevimab) was tested in a clinical trial in about 1500 healthy late-preterm and term infants and caused a 75% reduction in medically attended RSV-associated lower respiratory tract infection but unfortunately failed to show a statistically significant effect on hospitalization rates.[195]

One mAb has been approved for treatment of HIV infection, although it is directed to a host factor not to the virus. Ibalizumab is a humanized IgG4 mAb that binds to CD4 on host T cells and blocks virus entry. It functions as an entry inhibitor at a postattachment stage by blocking HIV binding to CCR5 and CXCR4 coreceptors. The mAb is used in combination with small molecule antiviral drug treatments, and in the phase III clinical trial, 59% of patients achieved viral suppression.[133]

The emergency period of the large West Africa Ebola outbreak in 2013-2016 led to the testing of new medical countermeasures against Ebola virus disease (EVD), including mAbs. An initial randomized controlled trial using a combination of three humanized murine mAbs to the Ebola glycoprotein (ZMapp) suggested benefit, but the study was inconclusive. In a subsequent outbreak of EVD in the Democratic Republic of Congo, where an outbreak began in August 2018, the Pamoja Tulinde Maisha (PALM) trial showed two mAb products (the three-mAb combination called REGN-EB3 [atoltivimab, maftivimab, and odesivimab-ebgn] and a monotherapy called mAb114 [ansuvimab-zykl]) demonstrated efficacy as compared to the ZMapp control arm.[316] In 2020, these mAbs became the first FDA-approved treatment for Zaire ebolavirus infection in adult and pediatric patients.

As of April 2022, four mAb products for SARS-CoV-2 had received Emergency Use Authorization (EUA) from the U.S. FDA; the issuance of an EUA does not constitute FDA approval but allows use during declared emergencies. Bamlanivimab plus etesevimab, casirivimab plus imdevimab, sotrovimab, and bebtelovimab received EUAs during 2021-2022 for the treatment of mild to moderate COVID-19 in nonhospitalized patients with laboratory-confirmed SARS-CoV-2 infection who are at high risk for progressing to severe disease and/or hospitalization. Distribution of bamlanivimab, bamlanivimab plus etesevimab, casirivimab plus imdevimab, and sotrovimab was ended because the products had reduced activities against some variants of concern. The FDA issued an EUA for the long-acting anti-SARS-CoV-2 mAb combination tixagevimab plus cilgavimab in December 2021, for using in preexposure prophylaxis in individuals who do not have SARS-CoV-2 infection, who have not been recently exposed to an individual with SARS-CoV-2 infection, and who are at risk for an inadequate immune response to COVID-19 vaccination or have a documented history of severe adverse reaction to an available COVID-19 vaccine.

Mechanisms of Antibody-Mediated Virus Neutralization

Abs may render free virus noninfectious, principally through neutralization of infectivity. Virus neutralizing activity typically is measured by *in vitro* assays in which Ab and infectious virus are incubated and then inoculated onto cell culture. Residual infectivity is quantified by plaque count, infectious foci marked by colorimetric or fluorescent markers, signal from recombinant reporter virus, percent of infected cells detected by flow cytometry, cell impedance measurements reflecting cytopathic effect, or other methods and compared to untreated suspensions. Viruses are neutralized *in vitro* by Abs by a wide variety of mechanisms. Important Ab characteristics include affinity/avidity, isotype, concentration, Ab to virus ratio, valency, state of polymerization, ability to bind the polyimmunoglobulin receptor (pIgR), ability to fix complement, ability to engage Fcγ receptors, and specificity for the virus protein and epitope of interest. Virus-related factors important to neutralization include general aspects of the replication cycle of the virus under consideration (receptor use, mode of entry into the cell, pH dependence of fusion, site of replication) and individual characteristics of the specific virus strain (antigenic subgroup and amino acid sequence of viral surface proteins). Host factors influencing the mechanism of neutralization include the type and origin of cells and the level of expression of membrane receptors. The most potent mechanisms of neutralization act prior to infection of cells. First, free infectious virions in solution can be aggregated by multivalent Abs directed against surface glycoproteins.[13,347] The consolidation of multiple infectious virions into an aggregate reduces the number of available infectious units. Second, Abs can inhibit virus attachment to cells, usually by binding directly to virus attachment proteins. Abs to attachment proteins often are poorly neutralizing *in vitro*, effecting only partial neutralization even at high concentration.[227] This effect may be caused by virus heterogeneity due to a variable extent of glycosylation that occurs during posttranslational processing at the numerous glycosylation sites on these proteins or because of multiple routes of entry or mechanisms of attachment. Third, viruses can be neutralized following attachment, at the cell surface prior to cell entry, suggesting that immune serum contains Abs capable of inhibiting virus-cell membrane fusion or other early steps of virus entry into the cell. For example, antibodies to HPV can interrupt infection at two stages of attachment and entry, the first an initial association with the acellular basement membrane that induces conformational changes in the virion and second the association of virus with the keratinocyte cell surface.[115] Some neutralizing Abs decrease infectivity by inhibition of virus-cell fusion at the plasma membrane. Fusion inhibition (FI) activity for some Abs has been inferred from *in vitro* assays in which Ab is incorporated into overlay medium after virus absorption and penetration, and activity measured by inhibition of postinfection cell-to-cell fusion. Additionally, Abs can reduce the release of progeny viruses from infected cells ("egress-inhibiting" antibodies). Antibodies to influenza neuraminidase (NA) surface glycoprotein are a prime example of such inhibitors, as many NA mAbs inhibit the enzymatic cleavage of sialic acid on the cell surface in the region of particle budding and release. An antibody to the "vestigial esterase domain" on the side of the influenza virus HA head domain also has egressing inhibiting activity, probably by tethering virus to the cell surface.[22] The influenza M2 ion channel protein participates in the formation and budding of new influenza A virions, and some antibodies that recognize the extracellular domain of M2 (M2e) inhibit the virus. M2e-based universal influenza vaccine candidates are being developed on this basis.[298] There is evidence that some influenza and Ebola virus–specific Abs inhibit virus replication by preventing the primary uncoating of virus that is associated with low-pH membrane fusion in the endosome or at the step of the secondary uncoating of the virion core structure, a step required for primary transcription of the virus genome. This principle was harnessed to create "Trojan Horse" bispecific antibodies that bind to viral or host proteins by one arm of the IgG to deliver the antiviral arm of the IgG for activity in the endosome.[481]

Structural and Biochemical Features of Antibodies Important to Affinity

Abs induced by primary infection or immunization are generally of low affinity for the target Ag. Affinity maturation of Abs is mediated by somatic mutation of the genes encoding B-cell receptors, which occurs in germinal centers, most prominently following second infections or booster doses of vaccine. SHM coupled with B-cell selection results in the promulgation of high-affinity Abs in the antiviral repertoire. Increases in affinity likely increase neutralizing potency for most antiviral Abs directed to surface proteins. Although the molecular basis for affinity maturation during secondary responses was not understood during the development of early vaccines, the enhanced efficacy of multiple-dose vaccine regimens in clinical trials pointed the way to a practical use of this principle. Most licensed viral vaccines are administered using

multiple-dose regimens to enhance the quality, magnitude, and durability of the immune response.

Increased affinity is widely believed to confer enhanced functional competence upon Abs. Work using HIV-1–specific Abs derived from combinatorial phage display Ab libraries demonstrated improvements in neutralization associated with affinity maturation caused by SHM.[456] The fusion (F) protein, the major protective Ag of RSV, has at least six major antigenic sites, each with a differing capacity as a target for virus neutralization.[32] Recombinant derivatives of a humanized RSV mAb containing random mutations in the combining site showed that mutations enhancing the association rate improved neutralization and efficacy, while mutations improving the dissociation rate did not, despite their contribution to improved steady-state affinity.[494] Somatic mutations in human rotavirus antibodies have been shown to enhance affinity and inhibitory function, with individual mutations in Ab combining sites conferring several hundred-fold increases in affinity and function.[227] The broadest and most potent human antibodies to HIV and influenza virus isolated to date are distinguished by exceptional numbers of somatic mutations in the Ab sequences (on the order of about 1/3 of nucleotides altered).[503,510] Hypermutation introduces not only point mutations that enhance function but in some cases nontemplated insertions of sequences encoding new amino acids that mediate neutralization.[250] In the vesicular stomatitis virus (VSV) mouse model, investigators have shown only modest increases in the affinities of hypermutated Abs compared with those in germ-line configuration over the course of infection and after repeated booster injections.[226] The VSV G glycoprotein is unusual in that it contains only a single immunodominant antigenic site that reliably induces neutralizing Abs. Many instances, however, have now been reported of neutralization by human antibodies lacking somatic mutations (created recombinantly by reverting inferred mutations to the germ line–encoded forms, termed reverted unmutated ancestor [RUA], or unmutated common ancestor [UCA] antibodies). Neutralization activity also is determined by the number of times the neutralizing determinant is displayed on the surface of the virion. HIV is interesting in this regard in that the number of envelope glycoproteins on the virus particles is remarkably sparse. Some studies suggest that certain types of virion particles may be dynamic in their display of epitopes.[124] The recognition of epitopes in the hidden interface regions of virus protein oligomers that are recognized by antibodies with antiviral activity implies a high level of dynamic excursion of the proteins in relation to one another. Examples of this pattern are the influenza HA trimer interface antibodies[21,478] and some neutralizing antibodies for henipaviruses.[121] Dengue viruses clearly exhibit “breathing” patterns involving the entire virus particle.[141] Understanding the relationship of affinity and neutralization is needed so that we can better appreciate the B-cell response to exposure with foreign Ags in a physiologic context. Furthermore, elucidation of the role of affinity in antiviral Ab function is critical for designing new active and passive immunization strategies against pathogenic human viruses. The absence of somatic mutations in the virus-specific Ab sequences of infants probably accounts for the difficulty in inducing high affinity high-potency Abs in this population after a single vaccination.[482]

CDR3 Loops of the Antibody Variable Region in Antigen Binding

A combining site formed by the variable heavy (V_H) and variable light (V_L) domains of the Ab fragment (Fab) mediates Ag recognition. Both the heavy and light chains have three noncontiguous linear sequences of greatest variability termed the CDRs (complementarity-determining regions) that result in flexible loops in the immunoglobulin (Ig) protein and form the classical Ag-binding site. The heavy-chain CDR3 (HCDR3) shows the greatest variability in terms of length and sequence, particularly in human Abs. Some crystal structures of Abs have demonstrated that human CDRs tend to adopt a limited number of canonical conformations, except for HCDR3.[80] In each of the crystal structures of human Abs complexed with Ags, the HCDR3 has been shown to make important contacts in every case.[25,429] Diverse CDR length and gene family usage have been shown to be essential features of immunologic health and functional antiviral immune responses.[167,246,417] Vaccinations that induce a broad Ab repertoire that is rich in CDR diversity are more likely to succeed than highly focused vaccinations inducing limited Ab repertoires. Although not universal, long HCDR3 regions are common in highly active Abs to HIV, and they may facilitate high-affinity binding to complex viral antigenic sites. For example, the crystal structure of the 17b mAb complexed with CD4-triggered HIV gp120 reveals a complex interaction that is accomplished only by the unique long HCDR3 region of this Ab.[255] High-throughput screening for broadly reactive high-potency HIV neutralizing Abs isolated two unusual antibodies with extremely long CDRs,[470] and the HCDR3s of these Abs possess fascinating secondary structural elements.[361] In contrast, identification of another broadly neutralizing mAb specific for the CD4-binding site has a CDR3 loop of only 13 amino acids, but the variable region of the heavy chains is more than 30% somatically mutated from germ line.[495] Many interesting sites of vulnerability for broad and potently neutralizing antibodies have now been identified, including antibodies that recognize the membrane-proximal external region (MPER), antibodies to the fusion peptide that blocks viral entry by inhibiting conformational changes in gp120 and gp41,[243] neutralizing CD4-binding site antibodies that mimic binding to CD4, and antibodies targeting the V3 glycan supersite on Env. Several mAbs have been evaluated in clinical trials including CD4-binding site antibodies VRC01 and 3BNC117 and V3 glycan supersite mAb 10-1074. The clinical approach to demonstrate benefit of antibodies in HIV infection involves testing in viremic persons infected with HIV-1 or in infected individuals during transient treatment interruption.

The Role of Isotype in Additional Mechanisms of Antibody-Mediated Virus Neutralization

The complement system is activated by the classical (Ab-dependent), alternative (Ab-independent), and lectin-binding pathways. Viral neutralization by specific Abs is concentration-dependent and is often enhanced by complement activation. It has been reported that even apparently “nonneutralizing” Abs can acquire neutralizing activity with the addition of complement, for example, a nonneutralizing RSV murine mAb was shown to exhibit complement-enhanced neutralization *in vivo* in the presence of complement.[101] Complement fixation is mediated by the C_H2 region of the Ab Fc domain of IgM, IgG1, and IgG3 subclasses in human and the IgM, IgG2a, and IgG2b subclasses in mice.[343] Understanding the role of complement in enhancing the functional capacity of Abs to neutralize viruses has broad implications for vaccine design, since the cytokine milieu induced by infection or immunization affects the isotype and subclass of the Ab response to the viral Ags by activating selective promoters in the appropriate Ig heavy chain locus. B cells triggered in an environment with high IFN-γ concentrations tend to produce IgG1 and IgG3 Abs. In contrast, in the presence of a dominant interleukin-4 response, B cells produce IgE and IgG4 Abs. IgE participates in allergic diseases, and it is generally considered desirable to avoid strong IgE responses following vaccination. The Fc region of Abs also mediates some cellular activities, termed Ab-dependent cell-mediated cytotoxicity (ADCC), which are discussed below.

Structural Features of Antigens Critical for Inducing Efficient Antibody Interactions

The conformation of epitopes on viral surface proteins often is critical to the ability of vaccine Ags to induce or bind neutralizing Abs. Most neutralizing Abs bind to conformationally dependent epitopes on surface proteins. Linear peptide vaccine candidates derived from the sequence of the natural Ag rarely recapitulate the conformation of the protective Ag, which is why most peptide-based vaccines are poor immunogens for

humoral immunity. Many subunit Ags comprising purified or recombinant viral proteins that have been developed as vaccine candidates contain the full polypeptide sequence of the Ag, but the protein emerges from the purification process in a conformationally relaxed state that resembles the natural Ag only in part. Such Ags may induce high levels of Abs that bind the vaccine Ag, but low levels of Abs that bind or neutralize virus.[409] Some viruses appear to present highly antigenic surface loops to the immune system that are structurally nonessential, causing immune focusing on hypervariable domains of viral proteins, such as the influenza HA variable loops surrounding the sialic acid receptor-binding domain (RBD) or the third variable (V3) region of HIV gp120. Some individuals make broadly neutralizing antibodies that recognize these hypervariable domains.[169] Many structurally critical domains of viral surface proteins are hidden from B cells because they are buried in the interior of viral molecules or oligomers of molecules, or they are exposed only briefly during rapid conformational changes required for virus attachment or virus-cell membrane fusion. For example, viral hydrophobic fusion peptides, RBDs, heptad repeats, and other critical regions of fusion proteins usually are not accessible to Abs. Most fusion proteins require cleavage by proteases before they are competent to fuse, and Abs to the precursor protein may not bind the cleaved protein in the mature virus particle.[352] Posttranslational modifications of surface proteins, especially glycosylation, can present a challenge for Ab recognition of protein because of the highly varied nature of the glycoprotein population. Deglycosylated protein Ags may enhance induction of protein-specific Ags, but these Abs may not efficiently recognize glycosylated proteins *in vivo*. Some virus neutralizing Abs are directed specifically to carbohydrates on virus glycoproteins, however.[61] Some broadly resulting antibodies to HIV, such as PG9 and PG16, recognize a conformational epitope that is dependent on glycosylation at specific variable loop N-linked sites.[123] Posttranslational modification of Abs also can affect the interaction of Ab and virus. For instance, several human Abs to HIV gp120 are sulfated in the combining site, which allows the Ag-combining sites to mimic the structure of the virus coreceptor CCR5, which is sulfated.[79] These Abs inhibit infectivity by binding to the coreceptor-binding domain on HIV gp120.

Antigens Recognized by the Humoral Immune Response

Development of an immune response to Ags present on the surface of virions or virus-infected cells is often critical for immunity against virus infection. Antibody (Ab) responses to surface viral protein or glycoprotein Ags often effectively limit infection of new cells and thus spread of the virus. The following generalizations can be made about the nature of Abs and their interaction with virus surface proteins. First, antiviral Abs against the extracellular domain of surface proteins predominantly recognize conformational epitopes that are often quaternary in nature, with extensive posttranslational modifications. Such conformational epitopes are difficult to mimic with peptides or proteins that have had the structure of antigenic sites modified by vaccine preparation and formulation. Presentation of the native Ag structure in its authentic conformation is critical for induction of neutralizing Abs. Even when the structure is mimicked with atomic level precision and elicits Abs that bind the epitope, the property of viral neutralization may not be achieved.[292,340] Most viral surface proteins assemble into oligomeric states on virions, typically dimers, trimers, or tetramers, but the monomeric form of the protein is also present in cell debris or shed proteins. The principal neutralizing determinant may be present only in quaternary structures, so that Abs binding to monomeric protein do not recognize or neutralize virions. In addition, many surface proteins that are targets of neutralizing Abs are fusion proteins that are present in a metastable state on virions. During the attachment and fusion process, the fusion proteins transition from prefusion to postfusion forms, in some cases drastically altering the structure of neutralizing epitopes. Recent protein engineering techniques have allowed rational design of recombinant viral surface proteins that are stabilized in a prefusion conformation to enhance immunogenicity, such as the prefusion RSV F protein candidate vaccine DS-Cav1.[364] Second, Abs recognize many major antigenic sites on each viral protein, and within each of these antigenic sites, there may be multiple epitopes. For example, the parainfluenza virus attachment glycoprotein (HN) has at least six antigenic sites, three of which are recognized by neutralizing Abs.[92] Antibodies to internal proteins that are not accessible on intact virions or to proteins that are present only in infected cells and not virions (nonstructural [NS] proteins) occur commonly. For some viruses, especially flaviviruses, antibodies to NS proteins can reduce or prevent disease, for example, antibodies to the NS1 proteins of West Nile virus or dengue virus. After infection, individuals often develop Abs only to a subset of the antigenic sites of the infecting virus.[93] After one or more reinfections, the Ab response usually broadens to recognize more antigenic sites. These findings provide a partial explanation for the failure of immunization to induce complete immunity in certain circumstances and provide a rationale for recommending multiple immunizations to achieve optimal protection. However, in the case of influenza, repetitive immunization or infection in a population may lead to narrowing of the recognition of virus variants due to immunodominance of certain antigenic sites.[515] This immune focusing is dangerous because frequent introductions of glycans on the surface of hemagglutinin (HA) can abrogate recognition of whole antigenic sites, which is especially unfortunate if the egg-adapted vaccines do not possess that same glycan.[514] The influenza NA protein also is susceptible to such major antigenic changes. For example, the NA of the circulating A(H3N2) viruses since 2016 are antigenically distinct from those in the prior vaccine strains due to amino acid mutations in NA introducing an N-linked glycosylation site.[472] Third, the large size of Ig molecules often precludes direct access to functional sites on viral surface proteins, such as the receptor-binding site, sites with enzymatic activity, or fusion peptides. Such domains can be buried within the protein structure of the surface protein or masked by carbohydrate molecules, variable loops, or conformational flexibility.[253] Consequently, the critical sites vulnerable to Ab-mediated neutralization can remain relatively inaccessible. Furthermore, some occult sites of vulnerability are hidden in the interface between multimer oligomers of a surface protein. Some highly conserved antigenic sites on viral surface glycoproteins are membrane-proximal, such as a class of bNAbs that target a discrete membrane-proximal anchor epitope of the influenza HA stalk domain[187] and/or similar epitopes on gp41 of HIV, the GP of filoviruses, and others. Therefore, Abs may exert their antiviral activities by mechanisms other than direct inactivation of functional sites on the surface proteins. Instead, Abs most often act directly or indirectly to impede attachment of virus to a host cell receptor, block virus fusion to the cell, or prevent uncoating of virus that has attached to or entered the cell. Unfortunately, effective Ab-mediated protection against virus infection is not always achievable. For example, the Ab response to the envelope glycoprotein of HIV evolves over time like the response elicited by repeated infections with parainfluenza virus, but the elaboration of the envelope-specific Ab repertoire is not able to keep pace with the rapid genetic evolution of HIV. The Abs present at a given point in time may neutralize virus isolated from the subject 6 months earlier but have very low neutralization activity against current strains that have mutated extensively.[390] Sufficiently high levels of passively administered broadly neutralizing mAbs can prevent simian-human immunodeficiency virus (SHIV) infection of macaques in some settings.[283] SHIV is a chimeric lentivirus with the internal and regulatory proteins of simian immunodeficiency virus (SIV), and the surface glycoprotein (gp160) of HIV-1. However, immunization with most currently available HIV-1 envelope antigens does not elicit Abs with these broad neutralizing properties.

Antibody Escape Mechanisms

Viruses, especially RNA viruses with error-prone RNA-dependent RNA polymerases, exhibit facility in generating genetic diversity during multiple replicative cycles. During chronic infections, such as those with hepatitis B or C viruses or retroviruses such as HIV, viruses that accumulate amino acid changes in epitopes that are targets of Abs due to point missense mutations in the corresponding genes can escape

neutralization by reduction or loss of binding. Some viruses easily shed surface glycoproteins (eg, HIV gp120 or Ebola GP)[203] or encode soluble forms of surface glycoproteins using alternative start codons (eg, RSV G protein),[391] in part as a decoy mechanism, targeting Abs away from infectious virions. The immunity induced by experimental vaccine candidates that are narrowly targeted to single epitopes or single proteins are susceptible to rapid viral escape. To avoid such Ab resistant mutants, optimal vaccines should induce broad humoral responses to a diversity of antigenic sites. The risk of narrow immunity is illustrated by the phase 3 trial failure of the RSV mAb suptavumab that failed to meet its primary end point of preventing medically attended RSV infections in infants in a study due to naturally occurring mAb-resistant antigenic subgroup B variant that circulated during the trial.[431] The suspension of EUA for therapeutic COVID-19 mAbs due to emergence of mAb-resistant variants of concern also exemplifies the risk of escape from narrow immunity, especially when RNA viruses circulate at large scale. So many variants have occurred during the COVID-19 pandemic; new vocabulary was developed by government interagency groups to organize discussion of the viral diversity observed and its impact. The CDC provides definitions[98] as follows: A *mutation* refers to a single change in a virus's genome that happens frequently, but only sometimes changes the characteristics of the virus. A *lineage* is a group of closely related viruses with a common ancestor. A variant is a viral genome that may contain one or more mutations. In some cases, a group of variants with similar genetic changes and shared characteristics may be designated by public health organizations as a *Variant Being Monitored (VBM)*, *Variant of Interest (VOI)*, *Variant of Concern (VOC)*, or *Variant of High Consequence (VOHC)* depending on the level of need for public health attention or action. Nextstrain is an open-source effort to display publicly available virus genome data for SARS-CoV-2, seasonal and avian influenza, Ebola virus, and chikungunya virus in real time coupled with tools for analysis and visualization (www.nextstrain.org). Viruses also may evade the action of neutralizing antibodies by accomplishing cell-to-cell transmission through passage in extracellular vesicles, exosomes, or nanotubes. In these structures, viruses may pass from one cell to another in a manner that is not accessible to recognition by antibodies.

Antibodies Active Against Virus-Infected Cells

Viruses that mature at the cell surface by budding or that insert viral glycoprotein(s) into the cell surface membrane render the infected cell susceptible to lysis by Ab-dependent complement-mediated lysis or Ab-dependent cell-mediated cytotoxicity (ADCC) mechanisms. This lysis is mediated by cytolytic cells including NK cells, neutrophils, and eosinophils, in an Fc region–dependent manner. Ab-dependent cell-mediated virus inhibition (ADCVI) activity is similar but is typically measured with a readout of reduction of virus replication instead of lysis of target cells. Abs that bind cell surface Fc receptors may arm leukocytes in the ADCC reaction, which has been shown to be important in the resistance of infant mice to herpes infection and for clearance of a retrovirus.[95,241] Fc receptor–mediated mechanisms appear important to neutralization of HIV[207] and many other viruses. Some studies show that the process of SHM is affected by the Fc glycan composition within antibody-antigen immune complexes, such as for antibodies recognizing the conserved stem region of influenza HA.[476] High-throughput methods for measuring surrogates of Fc-mediated functions have been developed to monitor the functions of antibodies, an approach sometimes dubbed systems serology.[83] These methods have been used to investigate the activity of nonneutralizing antibodies in vaccinees in the one human HIV vaccine trial that has demonstrated efficacy[84] and to suggest Fc-mediated effector functions also contribute to Ebola virus immunity in animal models, especially for mAbs with low neutralizing potency.[414] A challenge is how to validate that these putative CoPs function *in vivo* as they are modeled to do so *in vitro*.[267] There is currently a lack of standardization of Fc effector function assays in the field. Antibody effector functions have been shown to contribute to antiviral T-cell–mediated immunity against SHIV *in vivo*.[333] Some antibody Fc-mediated effects may enhance antigen presentation to T cells, inducing what has been termed a vaccinal effect.[48]

Some herpesviruses encode an IgG Fc receptor homolog, which enables the virus to evade ADCC by binding Abs, especially virus-specific Abs if they bind virus proteins on infected cells, suggesting the importance of the ADCC effector or other Fc-mediated mechanisms *in vivo*.[127] Since infection of cells must occur for these Fc-mediated immune mechanisms to act, these mechanisms represent a second line of defense against infection or disease.

Inadvertent Induction of Antibodies to Host Antigens

Immunization of monkeys with formalin-inactivated SIV produced in human cells induced Abs directed at Ags originating from the cells used to produce the virus.[435] These anticellular Abs can neutralize the infectivity of SIV produced in human cells, but not virus produced in monkey cells, by interacting with human Ags incorporated into the virion outer membrane. This form of neutralization, which is mediated by complement-mediated virolysis, can yield an overestimate of vaccine immunogenicity. Hence, an effort should be made to avoid immunization with virions into which foreign host-cell Ags have become incorporated.[435] In some cases, even the native virion proteins may mimic structures associated with host cell proteins. Polyreactivity (the property of binding to diverse structurally unrelated antigens) or autoreactivity (cross-reactivity to self-antigens) is commonly observed in the B-cell response to certain human viral infections like hepatitis C, Epstein-Barr virus (EBV), and HIV. For example, many of the original broadly neutralizing mAbs specific for the HIV-1 gp160 envelope glycoprotein cross-react with host cell proteins.[206] Certain HIV antibodies may exhibit low affinity of the antibody combining site for the HIV envelope but increase the avidity of the antibody by simultaneously binding with the second arm of the antibody to a host cell antigen, a process that has been termed heteroligation.[312]

Inhibitory Antibodies That Lack Classical *In Vitro* Neutralizing Activity

Some Abs that do not exhibit neutralizing activity *in vitro* can mediate resistance to viral infections, but typically, they are less effective than neutralizing Abs.[314] Such Abs can be directed against Ags on the surface of virus particles or against virion or nonstructural proteins displayed on the surface of infected cells. *In vivo*, these Abs mediate antiviral activities by several different mechanisms. First, virus particles coated with nonneutralizing Abs can undergo accelerated clearance from the bloodstream. Second, Abs directed at surface glycoproteins such as the NA of influenza A virus or the hemagglutinin-esterase protein of coronavirus can decrease the level of virus replication in the target organ, by inhibiting the release of virus from infected cells.[50,502] Third, Abs may lyse virus-infected cells via IgG Fc-dependent mechanisms.[101,419] Many antibodies to the nonstructural protein 1 (NS1) of diverse flaviviruses lack neutralizing activity, yet they protect *in vivo*; the diverse mechanisms mediating these protective effects are the subject of intense current research.[39,484]

Antiviral Activity of Antibodies *In Vivo*

Resolution of infection generally depends on cytotoxic T lymphocytes (CTLs), which reduce infection by lysing virus-infected cells. However, virus-specific Abs also can exert a therapeutic effect. Dramatic clinical therapeutic effect of viral Abs has been observed in patients with

Argentine hemorrhagic fever, which is caused by Junin virus, an arenavirus. This disease has a high mortality, but death can be prevented with a preparation of pooled human serum with a high titer of Junin virus neutralizing Abs administered within 8 days of onset of symptoms.[136,279] Mucosal virus infections that are limited to the cells that line the lumen of the respiratory tract also can be cleared by specific Abs that are delivered by parenteral inoculation or by direct instillation into the lungs. RSV immune globulin or RSV neutralizing Abs protect against severe lower respiratory tract disease.[349,387] These antibodies also reduce established infection in animal models, but therapeutic trials in humans have not revealed a clinical benefit of treatment of active infection. Influenza A virus hemagglutinin-specific IgG Abs cleared the lungs of influenza-infected SCID mice, in the absence of other recognized immune functions.[350] Abs can exert a therapeutic effect in privileged sites where CD8 CTLs are not active. Central nervous system neurons are not good targets for CD8 CTLs, because they have highly regulated expression of class I MHC glycoproteins. Nevertheless, in SCID mice, persistent infection of brain neurons mice by the alphavirus Sindbis virus can be cleared rapidly by parenteral inoculation of envelope-glycoprotein–specific Abs without causing obvious cell damage.[266] Evidence suggests that induction of IFN-γ may contribute to noncytolytic clearance of Sindbis virus from neurons by suppressing viral genomic RNA synthesis and reactivating cellular protein synthesis activation, leading to antiviral effects associated with activation of the Jak/Stat pathway. Some patients with underlying X-linked agammaglobulinemia complicated by chronic enteroviral meningoencephalitis have been treated successfully with human IgG that contains Abs specific for the infecting virus. A therapeutic effect was achieved with IgG administered by the intravenous or intraventricular routes.[130,293] Failure of human IgG to alter chronic enteroviral meningoencephalitis in some patients suggests that the concentration of enterovirus-specific Abs in human IgG is often inadequate. Under normal physiological conditions, the blood-brain barrier prevents most large molecules including antibodies from entering the brain.[23] Most biologics used to treat brain conditions are given intrathecally or they act in the periphery. New methods for delivering antibodies to the central nervous system are needed.

Mucosal Antibodies

Antibodies have been shown to function within mucosal epithelium.[286,287] Dimeric IgA secreted at the basolateral surface of cells or from plasma is transcytosed to the apical surface in association with polymeric Ig receptor to which dimeric IgA with J chain binds. During transcytosis through cells infected with the Sendai virus, virus-specific IgA can reduce intracellular virus and infectivity. The mechanism responsible for this effect is not clear, but it is possible that the endocytic pathway used for IgA transport and the exocytic pathways used for transport of viral glycoproteins intersect at some location within the cell, possibly the apical recycling endosome.[155] Similar studies with IgA Abs to rotavirus and measles virus suggest IgA molecules may inhibit viruses inside epithelial cells or after transcytosis.[54,498] Induced IgA molecules to HIV gp41 appear to block transcytosis across genital epithelial surfaces.[44] The FcRn receptor for IgG also is present in some epithelial cells and could participate in transport or retention of IgG at mucosal surfaces.

Antibodies present in the systemic circulation efficiently protect visceral organs against viruses that are introduced directly into the bloodstream (eg, HBV, HIV, etc.) or that spread via the bloodstream from primary sites of replication such as the respiratory or gastrointestinal mucosa. As mentioned above, diseases produced by viruses that fall into the latter category (measles, polio, hepatitis A, rubella, smallpox, and varicella) can be prevented or modified by Ig prophylaxis, even with concentrations of Ab that are so low they difficult to detect in the serum of recipients, and such Abs are relatively easy to induce by parenteral immunization with live or inactivated virus vaccines. To be fair, the efficacy of some parenteral immunization or direct immunization of mucosal surfaces with vaccines is often comparable in human trials, despite theoretical benefits of enhanced mucosal immunity from topical immunization, for example, for influenza or poliovirus vaccines. Nevertheless, Abs present in the systemic circulation may not provide efficient protection against viral diseases that are limited to mucosal surfaces unless these Abs are present in high titer. This is because only a small proportion of such Ig molecules traffic from the plasma to the luminal surface of the mucosal epithelium by transudation. Bronchoalveolar lavage (BAL) fluids have been reported to be contain about 1000 times lower levels of IgG than that in serum after systemic administration of IgGs. It is interesting that an Fc-engineered form of the RSV mAb motavizumab led to a 10-fold increase in *in vitro* FcRn binding at pH 6.0 for both humans and monkeys, and bioavailability in the lungs was also increased fourfold.[114] Therefore, to prevent viral diseases that are limited to mucosal tissue by vaccination, two different strategies can be employed. The first is to induce Abs such as secretory IgA at the mucosal site by virus replication or immunization at that site. The second, and less efficient, strategy is to stimulate a high titer of serum IgG Abs that can protect mucosal surfaces following transudation.

When IgG Abs present in the blood are passively transferred to mucosal surfaces by transudation where they can exert antiviral activity, there is a gradient regarding the ability of serum IgG-derived Abs to restrict virus replication on mucosal surfaces. This gradient is less in lung than it is in the nasopharynx. IgG Abs, if present in the serum in high enough titer, can provide almost complete resistance to pulmonary replication of RSV, but resistance in the upper respiratory tract is more difficult to achieve. Transudation of IgG also probably occurs in the intestine, especially in inflammatory states, but IgG is more easily subjected to proteolytic degradation in the intestinal lumen than in the respiratory tract. Immunization of the female genital tract with a live virus vaccine can induce the local synthesis of IgG antiviral Abs such that the specific activity of mucosal IgG Abs significantly exceeds that of serum, indicating that IgG Abs present on a mucosal surface can come either from serum via transudation or from IgG Ab secreting cells present in the submucosal tissues.[356] Although passively transferred IgG Abs can provide mucosal immunity in the lower respiratory tract, the major mediators of resistance to viral infection of the upper respiratory tract, the larger airways of the lungs, and the gastrointestinal tract are mucosal Abs, many of which are IgAs selectively transported across mucosal surfaces to exert antiviral effects on the luminal surface. Both nonneutralizing[54] and neutralizing[402] IgA Abs have antiviral effects on mucosal surfaces with the former active intracellularly during transcytosis and the latter active both intracellularly and on the luminal side of the epithelium. Antiviral IgA Abs function to clear viral infections, to modify the severity of disease on reinfection, and to prevent infection on re-exposure to virus. The level of antiviral IgA Abs and the number of virus-specific IgA secreting cells often correlates with resistance to viral infection.[89,505] The advantages of IgAs in the mucosal fluids are that they are transported more efficiently, they resist proteolytic degradation better than IgG, and the dimeric nature of secretory IgA enhances avidity.

The primary mucosal IgA response peaks within the first 6 weeks after infection and usually decreases to a low, often barely detectable, level by 3 months. The transient nature of the primary mucosal Ab response is a factor in the ability of many viruses to reinfect mucosal surfaces and underlies the need for two or more doses of vaccine to efficiently immunize mucosal surfaces. The virus-specific IgA Ab response is greater at the site of virus replication or of Ag administration than at distal sites, suggesting that mucosal immunity is best induced by antigenic stimulation of sites directly involved in replication of the wild-type virus.

Adverse Effects of Antibodies

Antibodies may contribute to the potentiation of disease. For example, heterotypic Abs to the envelope glycoprotein of dengue virus appear to

play a role in severe dengue.[248] Dengue viruses replicate primarily in cells of the mononuclear phagocyte lineage, and subneutralizing amounts of dengue virus Abs enhance dengue virus infection of these cells *in vitro* by increasing uptake of virus via IgG Fc receptors present on these cells.[194] However, the mechanism by which augmented disease results from enhanced access of dengue virus into mononuclear cells remains unclear.[400] Some model systems suggest enhanced dengue virus replication in the presence of nonneutralizing antibodies, and other studies suggest an association with enhanced cytokine production and vascular leakage. Immune complexes were detected in the lung tissues of fatal cases of influenza during the 1957 and 2009 pandemics, suggesting a potential role for enhancing antibodies in severe influenza disease.[304]

Vaccine-Induced Cellular Immunity

Major histocompatibility complex (MHC) class I–restricted CD8+ cytotoxic CTLs and MHC class II–restricted CD4 helper T cells (Th cells) each function independently as Ag-specific antiviral effector cells, and they also work cooperatively in most immune responses, for example, CD4 T cells often provide signals that facilitate optimal CD8 memory responses (see Chapter 10). Intact cellular immunity is critical for protection against progression of infection by some types of viruses. For example, most herpesvirus infections become more severe and destructive when cellular immunity is compromised. The attenuated virus vaccinia is used as the vaccine to prevent smallpox or monkeypox disease but also can cause progressive tissue invasion in persons with cell-mediated immune deficiencies or eczema. RSV and parainfluenza type 3 often become lethal infections in the setting of severe combined immunodeficiency or in patients who have undergone bone marrow transplantation. In contrast, patients with Ab deficiencies experience more severe disease only from selected viruses, particularly picornaviruses.

CD8 T Cells

CD8 T cells are the major T-cell effectors of antiviral activity. They are often referred to as CTLs, although they can clear virus-infected cells by both lytic and noncytolytic mechanisms. Classically, the CD8 T-cell receptor (TCR) recognizes a short peptide derived from an endogenously produced viral protein in the context of the MHC class I β2-microglobulin heterodimer expressed on the surface of an infected cell. Ag presentation by the MHC class I β2-microglobulin heterodimer is restricted to viral peptides that are produced and processed during infection. Therefore, the major CTL functions are to eliminate infected cells[146] or to inhibit virus replication by the elaboration of soluble mediators such as cytokines[151,317,455] or chemokines.[82,499] Dendritic cells (DCs) and some other professional antigen-presenting cells can take up extracellular protein antigens, process them as peptides, and present those with MHC class I molecules to CD8 T cells, a process that has been termed cross-presentation. Naive CD8 cells can be cross-primed in this way to become activated cytotoxic CD8+ T cells. Also, lymphocytes engaged in an immunological synapse with an antigen-presenting cell can capture membrane fragments containing surface molecules from presenting cells and express these molecules on their own surface, a process termed trogocytosis. The net effect of CTL activity is to prevent further spread of virus and to terminate infection in cells that are already infected. The importance of CTLs in recovery from viral infection is indicated not only by the association of cellular immune deficiencies with severe virus infection but by the diverse strategies that viruses use to escape from CTL immunity.[437] Viruses like herpesviruses, poxviruses, and lentiviruses have evolved strategies for interfering with Ag presentation pathways or CD8 T-cell effector molecules that compromise CTL activity. Lentiviruses are prototypes for escaping CTL killing by genetic variation and selecting mutations of key epitopes. CD8+ T-cell activity is critical for controlling SIV replication.[420] When that control is mediated by a dominant CTL response to a single epitope, immune control of viremia is lost when that epitope is altered by mutation.[26]

Although CTL antiviral activity generally is associated with the clearance of virus and the reduction of virus-associated pathology, disease enhancement can be observed under experimental conditions in which CTLs have been transferred passively to virus-infected recipients.[63,134,310] Therefore, one can view the immunopathology caused by the T-cell response to virus infection as the cost for virus clearance. A major goal of vaccination is to induce CD8 T cells with properties to clear virus-infected cells efficiently and rapidly to minimize the immunopathology associated with virus infections.

The time course of CTL activation during viral infection in the lungs is consistent with its important role in clearance of primary virus infection. Primary CD8 CTL activity in the lung peaks at about day 7 during acute virus infection, such as that produced by influenza A virus, LCMV, or RSV.[10,146,225] There is a subset of a long-lived memory T cells in mucosal and epithelial tissues with distinct phenotypes and specializations for the relevant tissues, and these have been termed tissue-resident memory T cells. These cells do not recirculate but persist in these extralymphoid tissues and provide protection against infection in those tissues. The naive CD8 T cell in the LCMV model undergoes a process of activation and expansion during that time of 10 000- to 50 000-fold.[42] After about 40 days, the level of detectable virus-specific memory CD8 CTL is about 10% of that during acute infection and is then maintained at constant levels for years even in the absence of Ag.[225] Each successive virus infection adds an expanded set of specificities to the pool of memory CD8 T cells over time and shapes the capacity of the host to respond to selected viruses.[425,426] The memory CD8 T-cell pool not only has an increased precursor frequency against virus-specific epitopes but those cells have a faster response time. The influence of CD8 CTL memory on the outcome of a subsequent virus exposure can be subtle and may depend on the absence or presence of other components of the immune response. For viruses in which a significant component of the clinical syndrome is related to the immune response clearing the virus, having memory CD8 T-cell responses will result in reduced virus replication a day or two earlier than in primary infection, an earlier peak illness time point, and a lower peak magnitude of illness.[151,179] In a setting where there is a large virus load (eg, high inoculum challenge) and an absence of preexisting neutralizing Abs to diminish the number of virus-infected cells, a large CTL response or infusion of excessive CD8 T cells can result in more severe disease.[62] During chronic virus infection, virus-specific CD8+ T cells may become exhausted as marked by upregulation of the inhibitory programmed death-1 (PD-1) pathway. Blockade of the PD-1 pathway in combination with therapeutic vaccination enhanced CD8+ T-cell immunity and resolution of chronic LCMV clone 13 infection in mice,[189] raising the possibility for a combinatorial therapeutic vaccination strategy to synergistically enhance epitope-specific CD8+ T-cell responses and accelerate viral clearance.

To date, the CD8 T-cell response has not been used successfully as a correlate of immunity that is the basis for licensure of a vaccine. However, the incorporation of CTL epitopes into viral vaccines has been considered valuable because of the important role CTLs play in clearance of established viral infections. The following characteristics of the CTL response *in vivo* should be kept in mind when designing a vaccine for viruses that may require a CTL component of immunity. First, the ability of a CTL epitope to induce an immune response is MHC dependent. Therefore, the incorporation of large antigenic content is important so that multiple CD8 T-cell specificities can be elicited by the vaccine to allow selection of epitopes by the diverse repertoire of human MHC class I alleles and to reduce the possibility of immune escape through genetic mutation. Second, when considering vaccine-induced immune protection, CTLs represent a second line of defense against virus infection because they cannot prevent infection and are only effective against infected cells. The localization and phenotype of CD8 T cells can influence

the timing and efficiency of viral clearance. For example, immunization of macaques with a recombinant CMV vector that induces a sufficiently high frequency of intraepithelial effector memory CD8 T cells that rapidly cleared virus-infected cells following SIV challenge leading to abortive infection.[200] Technologies such as polychromatic flow cytometry,[363] MHC class I tetramer reagents that provide the means to sort epitope-specific T cells,[119] the ability to define functional subsets of T cells with more precision,[116] sequencing technology that allows single-cell clonotyping of epitope-specific cells, and correlation of the TCR-peptide-MHC biophysical interaction and structure with T-cell functions[40] may provide the necessary information to harness CD8 T cells more effectively in the future. Indeed, investigators have developed methods using TCR sequences from peptide+ MHC-tetramer-sorted cells along with structural data to cluster TCRs with a high probability of sharing specificity, based on contact points with the antigenic peptides.[164] Impressive efforts have been made to standardize cellular immune assays that can be used in the context of clinical trials, for example, the IFN-γ ELISPOT assay for T cells.[218,278] The NIH has sponsored development of a publicly available curated comprehensive database of epitopes, MHC restrictions, and epitope prediction tools (The Immune Epitope Database and Analysis Resource [IEDB]) that facilitates such predictions.[411]

CD4 T Cells

CD4 T cells provide a variety of functions including help to B cells and CD8 T cells, thereby augmenting the two major effector mechanisms of the adaptive immune response. CD4 T cells can also mediate direct antiviral activity *in vivo*,[204,280,313,454] but this is not their major function as it is for CD8 T cells. The MHC class II, αβ heterodimer, which is the restricting element for CD4 T cells, is present predominantly on Ag-presenting cells such as DCs, macrophages, monocytes, and B cells. However, during inflammation even epithelial cells can express MHC class II molecules, and CD4 T cells have been shown to have compensatory viral clearance capacity in the absence of CD8 T cells. In addition to having antiviral and helper activity, CD4 T cells can mediate immunopathology[8,295,451] and have been shown to have regulatory and other specialized functions[512] (e-Fig. 15.5).

The CD4 T-cell response to certain internal viral proteins may play a cooperative role in the development of effective resistance by augmenting the Ab response to a major surface protein.[403] For example, immunization with the membrane (M) protein of influenza A virus can prime for a subsequent augmented Ab response to the HA glycoprotein on virus particles.[403] In this manner, the entire CD4 T-cell repertoire developed against the viral surface and internal proteins can be called into play to amplify a B-cell response to a surface protein. Because MHC class II–restricted T-cell epitopes tend to be more numerous on viral proteins than MHC class I–restricted epitopes,[251] the repertoire of CD4 T cells capable of augmenting the Ab response is large. While CD4 T cells are typically thought to provide indirect helper functions to improve CD8 T cell and Ab responses, there is evidence that they may also contribute direct effector functions to virus clearance. CD4 T cells have been shown to have cytolytic function against HIV.[345] Viral Ags can induce a protective immune response mediated by CD4 T cells in the absence of B cells or CTLs,[280] indicating that this arm of the immune response can make an independent contribution to resistance against both acute and chronic viral infections.[204] CD4 T cells also secrete IFN-γ, TNF-α, and other soluble factors that may directly inhibit virus replication.[338,339] For these reasons, it is advisable to include virus Ags capable of inducing CD4 T-cell responses in viral vaccines to achieve maximal immunogenicity.

The processes involved in CD4 T-cell memory induction differ from those involved in establishing CD8 T-cell memory responses[424] and are more complex in some ways. Ag processing and presentation in MHC class II molecules can occur through the endocytic pathway so unlike CD8 T cells, CD4 T cells can be induced by killed virus vaccines or even purified proteins and do not require live virus or gene delivery approaches to allow Ag to reach the cytoplasm. The zoster virus subunit vaccine induces CD4 T cells, which are thought to be the correlate of protection for this vaccine. CD4 T cells do not proliferate to the same extent as CD8 T cells during primary infection, and those producing IFN-γ do not survive as long-lived memory cells.[508]

CD4 T cells have traditionally been considered “helper” T cells (Th) and indeed their activities can improve the potency of B-cell and CD8 T-cell responses. Generation of high-affinity antibodies usually depends on cognate T-cell help based on physical contacts between T and B cells, mediated through interactions between the T-cell receptor and its cognate peptide presented on major histocompatibility class II molecules on the B-cell surface. Major subsets of CD4 T cells have been defined based on differentiation pathways and surface markers (e-Fig. 15.5). Th1, Th2, Th17, and Treg cell subsets express, respectively, the specific transcription factors: T-box expressed in T cells (T-bet), GATA3, retinoic acid–related orphan receptor gamma t (RORγt), or Forkhead box P3 (Foxp3). Th1 helper cells typically secrete effector cytokines IFN-γ and IL-2 that enhance cell-mediated responses performed primarily by macrophages and cytotoxic T cells. Th2 helper cells classically enhance humoral immune responses. In addition, to the original subsets Th1 and Th2, there are now many subpopulations of T cells defined with distinct functions, some of which can alter or diminish effector function. T regulatory cells (or Tregs) identified as CD4+FoxP3+ T cells have inhibitory effects on other immune effector functions, hence the name.[33] The extent to which vaccines induce CD4+FoxP3+ T cells with T regulatory activity is still poorly understood. Th17 helper cells are a subset of T helper cells distinct from the Th1 and Th2 lineages that produce interleukin-17 (IL-17). Th cells produce IL-17 and other proinflammatory cytokines that have been associated with immune-mediated pathology but are also important for immunity against some pathogens.[201] A CD4+CXCR5+ subset of CD4 T-cell–designated T follicular helper (Tfh) cells has been identified that resides primarily in lymph node germinal centers and promotes B-cell memory and plasma cell differentiation.[104] THαβ helper cells (triggered by IFN-α/β or IL-10) mediate antiviral immunity in some settings by secreting the effector cytokine IL-10 that alters the activity of many other immune cells. Newly defined T-cell subsets such as Th9 cells (an interleukin-9–producing T-cell subset) Th22 (interleukin-22–producing) cells occur, but their role in antiviral immunity is not clear. The role of each CD4 T-cell subset in vaccine-induced protection and optimal balance is an area that needs more investigation. Currently there is relatively little actionable information on how to control induction of optimal ensembles of CD4 T-cell subsets by vaccination.

Obstacles to Immunization in Special Populations

Immunization of Infants

The developmental immaturity of the immune response to viruses in the very young is seen clearly in the difficulty to immunize neonates and infants.[430] Young infants are a preferred target population for immunization, because most children present to health systems with the highest frequency at birth or shortly after. A high level of access to infants allows universal immunization, and many viral diseases are most severe early in life. Many obstacles arise, however, in attempts to immunize infants in the first several weeks or months of life against disease caused by viruses.

First, most infants possess maternal Abs that can interfere with response to infection or the infectivity or immunogenicity of primary immunization. Live attenuated measles virus cannot be given effectively prior to 1 year of age because many infants possess maternal Abs until that time that reduce or eliminate immunogenicity of the vaccine.[5] Ab-mediated suppression can be overcome in some instances by increasing the dose of the vaccine, for example, the inactivated hepatitis A vaccine or a replicating influenza vaccine.[90,111] The immune suppression by maternal Abs is more potent against B-cell responses than against induction of virus-specific T cells possibly because the Ab response is generally more dependent on Ag dose than T-cell responses.[106] Increasing dose of vaccine must be considered carefully, however. Measles vaccines that were 10-fold (medium titer) or 100-fold (high titer) greater than standard titer were given at 6-11 months of age in some African trials, and increased mortality was associated with high-titer vaccine for girls but not for boys.[210]

Second, the ability to respond to viral surface proteins with a protective Ab response is acquired gradually over the first year of life, reflecting developmental maturation of the neonatal immune system. Responses of seronegative infants to live measles vaccine remain relatively poor during the second 6 months of life.[157] Responses to live varicella-zoster virus vaccine also are reduced in the first year of life.[466] The youngest infants mount Ab responses to experimental mucosal immunization with live influenza, RSV, or parainfluenza virus vaccines that are low in magnitude, quality, and durability.[90,230,231] The molecular and cellular basis for immunologic immaturity is not fully defined. Infant virus-specific Ab sequences are characterized by marked lack of somatic mutations, resulting in low-affinity Abs of low function.[482] Infant B cells exhibit limited capability to switch isotype from IgM to IgG2 Abs, and the isotype profile of circulating Abs differs from adults until age 5-6 years. Infants possess reduced levels of some components of the complement cascade, especially C8 and C9 that participate in membrane attack complexes, leading to impaired complement-mediated reactions. Infants exhibit a high proportion of transitional immature B cells and poor organization of the splenic marginal zone.

The Ab responses that young infants make to respiratory virus surface glycoproteins are significantly lower in quality than those of adults. Studies have described a dissociation of the level of neutralizing and binding Abs in the very young infant.[493] This dissociation may be of functional significance since neutralizing Ab titers correlate well with protective efficacy in studies of protection for viruses. Dissociation between neutralizing and binding Abs also characterized the serum Abs induced by the ill-fated formalin-inactivated RSV experimental vaccine of the 1960s and probably accounts for the lack of protective efficacy of Abs induced by that vaccine candidate.[322] Ab responses induced by infections of infants are less durable than those of older subjects. Postinfection serum Ab levels to respiratory viruses often wane significantly several months after primary infection, especially if infection occurs at a very young age. Animal models suggest that B-cell priming occurs effectively early in life or in the presence of suppressive Abs,[105,106] but the deficient maintenance of long-lived plasma cells in the bone marrow may lead to rapid waning of humoral immunity.[365] Reduced humoral immunity to vaccines early in life also may reflect reduced T helper cell function and altered DC function. Neonatal CD4 T cells exhibit reduced ability to secrete IFN-γ, reflecting limited secretion of IFN-α and IL-12 by DCs.[117] Given the challenges of directly immunizing neonates against specific pathogens, some experts suggest integrated approaches using alternative strategies including vaccinating pregnant women and using vaccines that broadly alter neonatal immune status in a pathogen-agnostic manner.[242] For instance, the live attenuated bacille Calmette-Guérin (BCG) vaccine for tuberculosis has been much studied for its potential to induce heterologous beneficial effects that may protect against virus diseases. Current studies do suggest that BCG vaccination may affect ("train") innate immune cells in ways that alter some subsequent immune responses and metabolic and epigenetic reprogramming of those cells. But the heterogeneity of populations and their diverse environmental exposures makes it challenging to develop predictable and controllable effects of BCG for specific viral diseases.

Immunization of the Elderly

Obstacles to effective immunization also arise late in life due to immune senescence. Immune senescence in the elderly is marked by a decrease in the magnitude, quality, and duration of Ab responses and a reduction in functional T cells, especially CD8 T cells. T cells in the elderly are distinguished by alterations in cytokine production, costimulatory molecule expression, susceptibility to apoptosis, and changes in the level of DNA damage and repair and telomerase length.[158] Chronic virus infection may contribute to immune senescence. For example, CMV-specific T cells progress to a differentiated state in the elderly due to chronic antigenic stimulation.[359] This syndrome has been described as an "immune risk phenotype" in the elderly, reflecting the accumulation of a high percentage of circulating CMV-specific CD8 T cells at a terminal stage of differentiation that may detract from the flexibility of the T-cell repertoire to respond to other Ags. The CD4 T-cell responses of healthy elderly individuals appear to sustain a good level of function until late in life. Waning immunity to latent viruses may be boosted in the elderly in some cases. Immunization of the elderly with the zoster subunit vaccine reduces the incidence of shingles in that population.[108]

Aging has varying effects on the response to different Ags. For example, the immunogenicity of recombinant hepatitis B vaccine is reduced starting in the fifth decade and is poor in the elderly.[490] Ab responses to influenza virus vaccine also are reduced in the elderly. Practically speaking, however, while vaccination in the elderly may be reduced in efficacy and may require frequent boosters or other alterations in strategy, the effectiveness of many vaccines such as influenza virus vaccine are reasonable enough to support vaccination of this group. Higher-dose antigens have been licensed for use in those aged 65 years or older, and it also may be possible to improve the immune responses in the elderly by using new adjuvants or alternative vaccination strategies. Investigational gene-based vaccines have been shown to be highly immunogenic in older adults compared to the typical age-related decrease in responses seen with many traditional vaccines.[261]

Immunization of Pregnant Women

In the past, by default, pregnant women typically were excluded from vaccination programs due to safety concerns. However, immunization for many diseases has been shown to benefit both pregnant women and their children. Therefore, increasingly experts recommend the *presumption of inclusion* so that pregnant women would be included in vaccine development and deployment unless their exclusion can be justified from a scientific and ethical standpoint.[407] The CDC recommends that pregnant women get the inactivated influenza vaccine during every pregnancy. Pregnant people are more likely to suffer severe illness with COVID-19 compared with nonpregnant people and can receive a COVID-19 vaccine. Some vaccines should be given a month or more before pregnancy, such as the measles, mumps, rubella (MMR) vaccine, and hepatitis A and B are considered for pregnancies in which there would be risk for becoming infected with hepatitis viruses during delivery. Some vaccines are not recommended during pregnancy, including the MMR, HPV, live influenza, and varicella vaccines, and certain travel vaccines such as live yellow fever, typhoid fever, and Japanese encephalitis virus (JEV). It is generally safe to receive routine vaccines right after giving birth, even when breastfeeding, although yellow fever vaccine is not preferred in this setting.

Immunization of the Immunocompromised

Individuals with primary immunodeficiency or acquired immunodeficiency from immunosuppressive therapies face unique challenges with vaccines. First, there are increased safety concerns during vaccination for some individuals. Patients with phagocytic cell disorders, such as chronic granulomatous disease or with complement deficiencies can be immunized with standard protocols. However, people with T- or B-cell immunodeficiencies, such as severe combined immune deficiency, Bruton agammaglobulinemia, and common variable immune deficiency, may contract disease following inoculation with live virus vaccines such as MMR, varicella, oral rotavirus, live intranasal influenza, oral polio, or yellow fever virus. Immunodeficient patients also can acquire infections from close-contact spread following contact in public, home, clinic, or hospital with otherwise healthy subjects with infection or who are shedding live virus vaccine–derived strains. Patients who have received therapeutic hematopoietic stem transplantation are at exceptional risk prior to immune reconstitution and during periods when they receive immunosuppressive agents to prevent or treat graft vs host disease. These individuals should avoid live viral vaccines themselves. Second, immunodeficient patients often are unable to develop protective immunity following vaccination. For people with antibody deficiencies, Ig replacement therapy is the standard of care. Maintaining community ("herd") immunity in the population at large and especially in family members of patients with reduced T-cell and B-cell–mediated immunity protects these patients. COVID-19 proved a unique clinical scenario for testing options to protect immunodeficient individuals, as most people can safely get any of the three COVID-19 vaccines with emergency use authorization since they do not contain live virus. COVID-19 vaccination, including extra booster doses, was recommended for immunodeficient subjects. This population is quite heterogeneous, however, comprising individuals with primary vs secondary immunodeficiency, HIV infection, solid tumors or solid organ transplant, autoimmune conditions, and hematologic cancers (leukemia and lymphoma). A comprehensive approach to protecting these individuals includes vaccination and boosting, nonpharmacologic interventions to avoid exposure, and prophylaxis by passive immunization with a combination of two long-acting mAbs (tixagevimab/cilgavimab).

New Conceptual Approaches to Rational Vaccine Design—Structure-Based Vaccines

In recent decades, major advances in viral vaccine development have coincided with the emergence of new technologies (Fig. 15.4, e-Fig. 15.6A and B). Therefore, selected technologies that are likely to influence future vaccine design are discussed briefly.

FIGURE 15.4 **History of virus vaccines**. Viral vaccines can be organized by the major technology that made their development feasible. Licensed viral vaccines are listed on the left axis in chronological order. They are color-coded indicating the major development era or technology with which they were associated. Significant advances since the initial age of empiricism have been coincident with the development of cell culture techniques and molecular biology. One can envision that advances in some of the technologies discussed in the text such as structural biology, advances in sequencing, and gene expression vectors may facilitate vaccine development for unmet and unanticipated future needs for prevention of viral diseases.

e-FIGURE 15.6 Evolution of viral vaccine concepts. **A.** The progression of viral vaccine platforms has followed the availability of new technologies as shown in Figure 15.5. Live attenuated virus vaccine approaches were followed by inactivated virus vaccines. Subsequently, genes encoding specific vaccine Ags were derived from the virus of interest, first by extraction and now by using sequence information for *in vitro* synthesis. **B.** Impact of molecular biology on vaccine development options. Modern approaches to vaccine development virtually all use genes encoding viral proteins and molecular biology techniques to produce vaccine Ags and delivery vehicles. Licensed vaccines utilizing these approaches include VLPs for HBV and HPV and live attenuated virus vaccines for influenza and rotavirus.

Advances in Monoclonal Ab Isolation

The neutralizing determinants on vaccine Ags are *de facto* the epitopes recognized by human antibodies that mediate antiviral function. The study of the molecular and structural basis for neutralization was limited in the past by the difficulty in isolating human mAbs. Breakthroughs in this field using enhanced EBV transformation with CpG,[37] human hybridoma formation,[503] plasmablast sorting and cloning,[492] single B-cell sorting and expansion,[470] B-cell immortalization with lentiviruses transducing BCL6 and Bcl-xL,[252] massively parallel single B-cell interrogation in microfluidic devices,[513] LIBRA-seq,[427] and other techniques have increased the numbers of human antibodies for study by several orders of magnitude. Structural studies of complexes of human Abs with viral Ags have begun to yield fascinating insights into the features of protective Ab epitopes, which often are conformational, quaternary, and otherwise complex.[254]

Advances in Structural Biology Coupled With Computational Biology

The ability to design optimal antigenic surfaces for induction of neutralizing Abs has long been a dream of vaccine scientists. The goal is to develop synthetic constructs that elicit Abs with predetermined structural specificity. Induction of Abs against epitopes that are hidden in the native viral protein has been a challenge for vaccine design. Investigators working in the areas of structural biology and computational modeling have made significant progress in this regard. Computationally designed epitope-scaffolds that are immunogenic have been developed for the HIV-1 gp41 epitopes of the broadly neutralizing mAbs 2F5[340] and 4E10[102] and the RSV F motavizumab epitope.[292] Structural information from HIV gp120 CD4-binding site epitope recognized by the neutralizing Ab b12 has been used to design gp120 reagents to select additional CD4-binding site antibodies that have broad neutralizing activity against HIV.[510] Stabilization of the prefusion conformation of RSV F protein has led to a more immunogenic RSV vaccine candidate.[364] Fragments of influenza HA have been expressed to focus the immune response to conserved epitopes in the HA stem region.[43] The NIH Vaccine Research Center is testing a vaccine candidate composed of the HA stem domain from influenza A/New Caledonia/20/l999 (H1N1) genetically fused to the ferritin protein from *Helicobacter pylori*, which displays eight HA trimers that resemble the native HA stem region.[501]

Computational prediction tools are being used to design vaccine candidates with optimal properties. The Rosetta molecular modeling suite, maintained and developed by the RosettaCommons international collaboration, has been used extensively in docking and design application cases for antibody and antigen modeling.[422] Both side chain and backbone grafting have been applied successfully in multiple cases to place epitopes into scaffolded antigen frameworks. Computational and structural tools also can be used for design of viral inhibitors, for example, the design of proteins targeting the influenza HA stem that mimic the action of neutralizing Abs.[143] Small (<65 amino acid) protein designs that bind their targets with nanomolar to picomolar affinities have been designed using only information in the three-dimensional structure of influenza HA or SARS-CoV-2 spike proteins.[64] AlphaFold, a deep learning system based on an artificial intelligence (AI) program developed by

Alphabet's/Google's DeepMind, performs predictions of protein structure with increasing power to predict or design structures.[224] These computational design methods are optimal for propelling the field of rational vaccine design, which incorporates the concepts of antibody germline targeting and epitope-focused and antibody lineage–based immunogen design.[55] It seems likely that computationally designed immunogens will increasingly influence viral vaccine design.

Sequence-based methodologies are also being used for design of more broadly immunogenic antigens, for example, using the COBRA (computationally optimized broadly reactive antigen) method that has been used for sequence consensus building to generate influenza vaccine HA immunogens for H1, H3, and H5 influenza subtypes.[7] Some HIV vaccine candidates have been designed *in silico* to target multiple viral subtypes using what are termed recombinant mosaic antigens, which constructed from natural sequences to include common broadly protective conserved epitopes while reducing rare sequences, which appear more broadly protective in nonhuman primate studies.[28]

Types of Vaccines

Live Virus Vaccines

History of Methods for Isolating Attenuated Strains

Varying methods have been used to achieve attenuation for live virus vaccines. Type 2 poliovirus, strain 712, one of the three poliovirus vaccine strains in the live vaccine preparation, is a naturally occurring attenuated human poliovirus that was identified by its lack of virulence for the brain and spinal cord of monkeys.[405] Adenovirus vaccine strains (types 4 and 7) are wild-type human respiratory viruses that were developed for use in the U.S. military that produce an asymptomatic infection following oral administration that was restricted in replication to the intestinal tract.[73] The remaining attenuated vaccine viruses were derived from wild-type human viruses by serial passage in cell cultures prepared from an unnatural host, leading to the emergence of mutants that were partially restricted in humans at the portal of entry and/or the target organ(s). In this manner, attenuated mutants of rubella virus and types 1 and 3 poliovirus were selected after passage in monkey kidney tissue culture. Vaccine strains of yellow fever virus (17D strain) and measles virus were generated by multiple passage using cells in tissue from embryonated chicken eggs, whereas mumps virus was attenuated by multiple passages in embryonated eggs. Vaccinia virus was used to eradicate smallpox, but after eradication, its safety profile was considered suboptimal. A highly attenuated derivative strain of vaccinia virus called modified vaccinia Ankara (MVA) virus was developed by more than 500 serial passages in chicken embryo fibroblast cells of a wild strain of vaccinia isolated by the Turkish Vaccine Institute of Ankara. MVA lost ~10% of the vaccinia genome and consequently the ability to replicate efficiently in primate cells,[12] but it is highly immunogenic in humans.

A critical feature of most successful live vaccine development programs is the identification of preclinical laboratory predictors of attenuation. For the yellow fever virus and poliovirus vaccines, promising vaccine strains were identified by their attenuation for experimental animals. In monkeys, the vaccine based on the 17D strain of yellow fever virus exhibited decreased tropism for the liver, suggesting that this mutant might be suitably attenuated for humans,[452] a hypothesis that was confirmed during subsequent clinical trials. Naturally occurring or tissue culture–passaged strains of poliovirus were evaluated for neurovirulence in monkeys by intraspinal inoculation, a central innovation that allowed Sabin to identify attenuated strains.[404,405] This testing system was selected because several observations suggested that the spinal cord of monkeys was more permissive than that of humans to the neuronolytic effect of poliovirus.[405] Viruses that caused the least neurovirulence in monkeys were identified, and these strains then were cloned and recloned biologically by the plaque technique to yield progeny with the lowest possible level of monkey neurovirulence. These candidate vaccine strains then were subjected to additional cycles of selection, which led to the identification of mutants that replicated with high efficiency in the intestinal tract without significant increase in neurovirulence for nonhuman primates. These viruses were evaluated in clinical trials and shown to be satisfactorily attenuated and immunogenic.

Experimental animal systems were not available for evaluating virulence of the other vaccine viruses. Therefore, these viruses were tested for attenuation directly in humans. Initial candidate measles and rubella virus vaccines were insufficiently attenuated, but further passage of rubella virus in monkey kidney cell culture and selection of a cold-adapted, temperature-sensitive mutant of measles virus yielded satisfactory vaccine strains. Attenuation of mumps virus passaged in eggs also was determined in humans. Interestingly, the Jeryl Lynn strain of mumps virus vaccine developed by Maurice Hilleman as a clinical isolate from his daughter was found to be a mixture of two closely related strains of mumps virus.[2] Several other vaccines have been found to be mixtures of virus strains, including the yellow fever virus vaccine and some vaccinia stocks used for smallpox vaccination.

Poliovirus and adenovirus vaccines are administered orally, while yellow fever, rubella, measles, and mumps virus vaccines are given parenterally. Each of the currently licensed live virus vaccines is directed against a virus that has a complex pathogenesis of infection in which virus is introduced by inoculation (yellow fever) or by implantation on a mucosal surface (measles, mumps, rubella, and poliovirus) with subsequent spread systemically to the target organ(s). The requirement of such viruses to pass through blood to reach the target organ of disease makes them generally susceptible to serum antibodies. It is more difficult to protect against infection by viruses that cause disease at a mucosal portal of entry without need for viremia.

Advantages of Live Virus Vaccines

The major advantage of live virus vaccines is the activation of all components of the immune system yielding both a balanced systemic and local immune response and a broad humoral and cell-mediated response. This type of comprehensive induction of immune effectors is particularly important for infections in which cell-mediated immunity plays an important role and for mucosal infections in which both local and systemic immunity are required for optimal resistance. As noted, mucosal infection with a live virus vaccine is much more effective in stimulating a local response in the unprimed host than inactivated virus vaccines administered parenterally.[319] Live virus vaccines also can stimulate an immune response to each of the protective Ags of a virus, and this obviates the difficulties that arise from selective destruction of one of the protective Ags that may occur during the inactivation process and can lead to disease potentiation. This advantage is especially important for complex viruses encoding many protective Ags since it is prohibitively difficult and expensive to immunize with multiple purified proteins or to construct multivalent vectors. Furthermore, immunity induced by live virus vaccines is generally more durable and more effective.[220] There are practical advantages to many live virus vaccines, such as relatively low cost of production and ease of administration. Live viruses contribute to protection in populations because of the induction of herd immunity, sometimes facilitated by transmission of vaccine viruses from a recipient to a contact. The effectiveness of live vaccines in settings of high transmission is illustrated using adenovirus vaccines. Adenovirus vaccines reduced military

respiratory disease morbidity significantly over the last three decades. In 1995, however, the manufacturer of these vaccines ceased production due to budgetary constraints. Major outbreaks of serious adenovirus infections occurred in military facilities, therefore.

Disadvantages of Live Virus Vaccines

The major concerns with live virus vaccines are that they (1) can contain adventitious agents, (2) can cause illness directly, (3) can lose their attenuation during manufacture or during replication in vaccinees by reversion or by second-site compensatory mutations, (4) can spread to contacts, and (5) can lose infectivity during storage, transport, or use. The potential for contamination with live adventitious agents always exists as discussed above. Sources include the original clinical sample from which the virus was isolated, cell culture substrates, laboratory reagents of animal origin (such as serum supplements and trypsin), and contamination from laboratory workers or environments. Cross-contamination of vaccine lots with other viruses in a laboratory facility is possible. Fortunately, contamination of clinical lots of vaccine with major human pathogenic adventitious agents has rarely been a problem in the modern era. Some of the early lots of live poliovirus vaccine were contaminated with live SV40, and the live yellow fever virus vaccine was initially contaminated with an avian leukosis virus. Follow-up of individuals who were given live vaccine contaminated with SV40 or avian leukosis virus has failed to identify any long-term adverse effect, including cancer, associated with exposure to these adventitious viruses.[308,441,479]

Some live virus vaccines, such as the measles virus, rubella virus, and yellow fever virus vaccines, retain a low level of residual virulence. The reactions produced by these vaccines generally are minor and have not interfered with acceptance and widespread use of these products. A more serious problem is that of restoration of a varying degree of virulence during infection by vaccine virus. This occurs with the poliovirus vaccine at an extremely low frequency, that is, about one in 10^6 to 10^7 immunizations.[334] Most vaccine-associated cases of paralysis in vaccinees or their close contacts occur after the first dose and involve the type 3 vaccine strain.[334] A significant proportion of the paralytic illnesses associated with poliovirus vaccine occurs in individuals who are immunocompromised, but this may not represent a manifestation of genetic alteration of vaccine virus.[334]

The genetic basis for the very rare increase in virulence of the poliovirus vaccine strains during replication *in vivo* is relatively well understood. However, a paradox exists in that mutation toward a higher level of virulence for the CNS occurs rapidly and frequently during replication of poliovirus in the intestinal tract of vaccinees. During manufacture of vaccine, the mutations that are associated with restoration of virulence can be detected at variable low frequency by sequence analysis, and neurovirulence of the final product can be held to a low level by rejecting vaccine lots that exceed the accepted standard for frequency of these mutations.[81] Genetic instability was also a problem with the 17D yellow fever virus vaccine during early field trials. Encephalitis was observed in 1%-2% of young vaccinees administered virus that had been passaged 20 times beyond the original seed lot.[149]

The problem of genetic instability of live attenuated virus vaccines following replication *in vivo* is an even larger concern for viruses that can cause latent or persistent infection *in vivo* such as herpes viruses or lentiviruses, for example, HIV. Despite this concern, successful vaccines have been developed for VZV, a herpes virus that causes chickenpox and zoster, indicating that a successful live attenuated vaccine can be developed for a virus that causes a lifelong infection in humans. This vaccine was licensed first for prevention of childhood chickenpox and was shown to significantly reduce the risk of VZV reactivation or shingles.[348]

Rubella virus can be recovered from the lymphocytes of immunologically normal individuals with arthritis up to 6 years after immunization or natural infection,[74] but the rubella virus vaccine does not appear to be associated with chronic arthritis.[385] Interestingly, persistence of measles vaccine virus has not been detected nor has the vaccine virus been implicated in subacute sclerosing panencephalitis.[297] Instead, widespread use of live measles vaccine has almost eradicated this rare but serious sequelae of persistent wild-type measles virus infection.

Several other concerns have been identified for live attenuated virus vaccines. First, there is a theoretical risk that infection of the fetus could occur following vaccination during the first trimester of pregnancy. For rubella vaccine, there is concern that vaccination might lead to development of the congenital rubella syndrome; however, the actual risk appears to be negligible.[355] Second, naturally occurring wild-type viruses may interfere with infection by a live virus vaccine resulting in a decrease in vaccine efficacy. Such interference has been observed primarily with live poliovirus vaccine strains and is caused by a wide variety of enteric viruses. This phenomenon has also been seen with experimental live attenuated alphavirus vaccines.[288] To overcome this viral-viral interference, multiple doses of polyvalent live virus vaccines are required to assure a protective immune response to each component. Third, defective interfering particles have been identified in preparations of live virus vaccines,[60] and it is likely that they are present in all live virus vaccines to some degree. Fourth, live attenuated measles virus vaccine has been associated with a generalized immunosuppression that was observed to be directly correlated with the level of immune response to the vaccine.[215]

Finally, it should be noted that stability is a serious practical problem with thermolabile vaccine viruses such as measles virus. The need for storage and transport of measles vaccine at low temperature (4 °C) has limited its usefulness in some tropical areas where maintenance of a "cold chain" for transport and storage is difficult. Some simple solutions for stabilizing other live virus vaccines have been identified such as lyophilization of vaccinia virus or using deuterium oxide for water in live poliovirus or influenza vaccine formulations,[216,300] but additional work is needed.

Genetic Basis for Attenuation of Live Viruses

Mutants selected by passage in an unnatural host accumulate many mutations, often making it difficult to define in a precise manner the genetic basis for their attenuation. In some cases, the molecular basis of attenuation has been defined. The genetic basis of this neuroattenuation of each of the three poliovirus vaccine strains has been partially defined.[182] Surprisingly few mutations are involved. Analyses have identified attenuating nucleotide substitutions in 5′ noncoding sequences and other attenuating in the 3′ noncoding region or in the structural or nonstructural coding regions of type 1, type 2, or type 3 polioviruses.[47,160,389] These mutations can now be monitored during manufacture and following replication *in vivo*. Vaccine lots with an increased frequency of reversion at some residues have been shown to exhibit increased neurovirulence, and such lots can be discarded as unacceptable for human use.[81] Thus, it is now possible to use molecular virological techniques to control the manufacture of vaccines by identifying the presence of the desired attenuating mutations as well as the absence, or low frequency, of unwanted reversions.[81]

The Jennerian approach to the development of live attenuated viruses involves the use of a virus strain of mammalian or avian origin to immunize humans against a human virus that is related antigenically to the animal or avian strain. Mammalian and avian viruses that are well adapted to their natural host often do not replicate efficiently in humans and hence are attenuated. This host-range restriction of course was the original principle used by Jenner for the first vaccine. At present, we lack a complete understanding of the genetic basis for this form of host-range restriction. However, those mammalian or avian viral genes that have been identified as being responsible for host-range restriction in humans exhibit significant divergence of nucleotide sequence from that of the corresponding human viral genes. Quadrivalent and pentavalent rotavirus vaccine are examples of the Jennerian approach to vaccine development in which a nonhuman rotavirus strain, the rhesus rotavirus (RRV) or bovine rotavirus (BRV), were found to be attenuated in humans. Since there was a need for a multivalent vaccine that would induce resistance to each of the major human rotavirus serotypes, the Jennerian approach was modified by constructing three reassortant viruses, each of which contained 10 RRV or BRV genes plus a single human rotavirus gene encoding one of the major neutralization Ags of the targeted serotypes.

Conventional techniques for attenuation of viruses such as passage of virus at low temperature, mutagenesis followed by selection of mutants with the desired phenotype(s), or passage of virus in heterologous tissues continue to play a significant role in the development of live virus vaccines. These techniques have been used to generate candidate vaccine strains for CMV, ortho- and paramyxoviruses, alphaviruses, flaviviruses, HAV, and arenaviruses. Selection for a combination of phenotypes (such as cold-adapted [*ca*] and temperature sensitive [*ts*]) is thought to be desirable because the different mutations can have synergistic effects on the stability of the attenuation phenotype *in vivo*.[321]

The function of viral proteins can be altered by missense mutations, insertions, or deletions. Classically, attenuated viruses were identified using biologic phenotypes: (1) *ts* mutants, (2) cold passaged (*cp*) or cold-adapted (*ca*) mutants, (3) small plaque mutants, (4) protease activation (*pa*) mutants (also designated as cleavage mutants), and (5) mutants with altered interaction with host cell receptors. With modern molecular techniques, virulent viruses also can be attenuated by a wide variety of creative approaches including gene deletion, shuffling of gene order, alteration of noncoding regulatory regions contain cis-acting signals required for efficient replication, use of gene incompatibility in chimeric genomes, and the insertion of genes that encode proteins with known antiviral activities or with known immunoregulatory functions. Viruses bearing stable, molecularly defined, identifiable attenuating mutations or attenuating gene constellations represent the vaccine strains of the future because the genetic basis for attenuation will be known and can be monitored directly during all phases of vaccine development, manufacture, and use in humans. An interesting synthetic approach to attenuating viruses uses synthesis of nucleic acid copies of viral genomes that alter the genetic code using synonymous codons, thus increasing the number of suboptimal codon pairs.[94] This method has been termed *codon pair deoptimization*. Suboptimal codon pairs cause attenuation by reducing mRNA stability and translation efficiency and thus reducing the amount of viral protein produced.[181]

Inactivated Virus Vaccines

Inactivated virus vaccines are available in the United States for the prevention of disease caused by nine separate viral agents (Table 15.1). The available vaccines are based on either whole inactivated virus or viruslike particles (VLPs) produced from purified protein. The viruses are grown in a variety of cell substrates including eggs (influenza types A and B), a continuous monkey kidney cell line (poliovirus types 1, 2, and 3), human diploid fibroblasts (rabies, hepatitis A), or mouse brain (JEV vaccine). Virus is then inactivated with a chemical such as formalin or disrupted with a detergent (influenza). The level of efficacy of inactivated viral vaccines differs—inactivated poliovirus vaccine is highly effective in preventing disease, whereas influenza virus vaccine is only partially protective.

Features of Inactivated Virus Vaccines

Inactivated virus vaccines offer the advantage of immunization with the entire antigenic content of the virus with little or no risk of infection. Only rarely do such vaccines contain a contaminating adventitious agent or residual infectious virus that has resisted inactivation. For example, paralytic disease was produced by some of the early lots of inactivated poliovirus vaccine that contained residual infectious virus.[328,341] Contamination by an infectious adventitious agent was also detected retrospectively in some early lots of inactivated poliovirus. This was of some concern because the contaminating simian virus, SV40, was oncogenic in hamsters.[211] Fortunately, long-term follow-up of individuals who were inoculated with SV40-contaminated vaccine during early infancy failed to show evidence of a vaccine-induced oncogenic effect.[441] Another incident occurred in a research setting where live vaccinia virus was found to be present in a preparation of inactivated vaccinia-HIV recombinant virus being evaluated as a therapeutic vaccine in HIV-infected subjects. Unfortunately, an immunodeficient recipient of this vaccine developed progressive vaccinia caused by residual live virus in the inoculum.[506] Several inactivated whole virus vaccines have potentiated disease rather than prevented it.[156,235] This finding was first observed with formalin-inactivated measles virus vaccine.[156] Initially, this vaccine prevented measles, but after several years, vaccinees lost their resistance to infection. When subsequently infected with naturally circulating measles virus, the vaccinees developed an atypical illness with accentuated systemic symptoms and pneumonia.[156,327] Retrospective analysis showed that formalin-inactivation destroyed the ability of the measles fusion (F) protein to induce hemolysis-inhibiting Abs, but it did not destroy the ability of the H (hemagglutinin or attachment) protein to induce neutralizing Abs.[336,337] When the H-specific Ab had waned sufficiently to permit extensive infection with measles virus, an altered and sometimes more severe disease was seen at the sites of measles virus replication.[58] This atypical disease is believed to be mediated in part by an altered cell-mediated immune response in which Th2 cells[376] or a delayed-type hypersensitivity reaction[264] were induced preferentially leading to heightened disease manifestations at the sites of viral replication. Immune-complex deposition also plays a role in the pathogenesis of atypical measles.[377]

Potentiation of disease also was observed after parenteral administration of an experimental formalin-inactivated RSV (FI-RSV) vaccine.[235] In clinical trials conducted in the mid-1960s, an FI-RSV vaccine induced a measurable serum neutralizing Ab response but did not protect the youngest vaccinees. Unexpectedly, upon subsequent natural infection with RSV, the FI-RSV vaccinees developed severe RSV lower respiratory tract disease significantly more often than did infants and young children who had received an inactivated parainfluenza virus vaccine.[235] Speculation on the mechanism of disease potentiation by the inactivated RSV vaccine has centered on several possible aberrations of the immune response to vaccine that involve an imbalance between or within various compartments of the immune system. Most likely, the protective antigenic sites on the F protein were altered by fixation, and the altered Ag induced an aberrant T-cell and cytokine response leading to enhanced disease.[171] The major lesson learned from the FI-RSV vaccine–enhanced illness is that the chemical treatment and physical purification procedures used for the preparation of each new inactivated vaccine should be evaluated not only for their effect on the magnitude of immune responses but also on the functional characteristics of the response. Typically, the Ab induced should have neutralizing activity, and the T-cell responses should mediate efficient virus clearance.

Potentiation of disease by an inactivated virus vaccine is not limited to vaccines containing measles virus or RSV and has occurred with both human and veterinary vaccines. For example, administration of an inactivated caprine lentivirus vaccine has also been associated with an accelerated, more severe disease in animals subsequently challenged with virus.[291] Vaccine-enhanced pathology is also not limited to inactivated virus vaccines. Immunization with a subunit vaccine of another lentivirus, equine infectious anemia virus (EIAV), enhanced disease caused by subsequent challenge with a heterologous EIAV strain.[217] A similar phenomenon occurred during the development of a vaccine for feline infectious peritonitis virus (FIPV). FIPV is a coronavirus (CoV) that developed more notoriety after the emergence of the SARS-CoV in 2003. Attempts to develop a vaccine against FIPV have been largely unsuccessful and have been associated with a vaccine-enhanced illness. A major problem is that Abs are not protective even when given passively for prophylaxis and can facilitate virus entry through Fc receptors. Whole virus,[360] recombinant vaccinia expressing the major surface S (spike) glycoprotein,[467] or DNA immunization with IL-12[163] all have led to enhanced disease syndromes. Immunization of ferrets with a recombinant MVA expressing the S glycoprotein has also caused an enhanced disease with hepatitis after SARS-CoV immunization. The immunologic mechanisms responsible for these examples of enhanced disease have not been identified. When SARS-CoV-2 emerged as a pandemic virus, rapid decisions had to be made about the potential for vaccines or antibodies to cause antibody-dependent enhancement (ADE) of disease. Natural infection and vaccination with immunogenic vaccines protected against disease in humans, without detectable enhancement of infection, virus replication, or disease. A thoughtful systematic review of research on ADE was published during the COVID-19 pandemic.[15]

Another disadvantage of inactivated virus particle vaccines is that they typically must be given parenterally because without replication or gene expression, it is difficult for mucosally delivered Ag to induce strong responses. Parenteral induction of systemic immunity may not fully protect against viruses that infect and cause disease primarily at mucosal surfaces if the gradient of Ab between serum and mucosa is too large or the kinetics of viral entry are too rapid. Therefore, resistance to viral challenge in the upper respiratory tract or in the intestines usually is less robust than that conferred by local immunization or infection. This may be the reason inactivated influenza vaccines are only partially protective, and why the IPV can prevent paralytic polio but cannot prevent poliovirus infection and intestinal carriage. There are clear exceptions to this principle, best exemplified by the success of parenterally administered HPV vaccine. Although HPV is a purely mucosal pathogen, entry into basal epithelial cells is a two-step process that may take several hours.[238] Virus neutralization can be accomplished with much less Ab when given longer access to the viral antigenic sites.

Inactivated virus vaccines are clearly at a disadvantage compared to either live attenuated virus vaccines or vector-based gene delivery with respect to induction of a CTL response. CD8 CTL responses require Ags to be processed through the proteasome so that peptides can be transported into the endoplasmic reticulum and associate with MHC class I molecules. This is best achieved when proteins are synthesized in the host cell cytoplasm and is rarely achieved in primates when Ags enter the APC through the endocytic pathway. For example, inactivated influenza virus is considerably less effective than virus infection in stimulating a primary CTL response in mice. However, inactivated influenza A virus or purified surface glycoproteins derived from it can sometimes stimulate a secondary CTL response in sensitized mice or humans.[1,135] There are new adjuvants and delivery vehicles that have the capacity for moving purified proteins into the MHC class I presentation pathway, and these may be considerations for future vaccine development.

The Guillain-Barré syndrome (GBS) was associated with widespread use of inactivated influenza A H1N1 (swine) virus vaccine during 1976 and 1977.[258] GBS does occur rarely in the population, but the attributable risk was estimated at one additional case of GBS for every 100 000 doses administered. This finding suggested that unanticipated, delayed, or untoward side effects can be induced by inactivated virus vaccines. This syndrome has been associated only rarely with subsequent influenza virus vaccines. Most studies show no association. But two studies since 1976 reporting positive findings suggested a risk estimate of about one additional GBS case per 1 million persons vaccinated, and this risk has been stated by the Advisory Committee on Immunization Practices recommendations. The occurrence after use of vaccines against the 2009 H1N1 pandemic strain also is negligible, even though these products were prepared in the same manner as the 1976 vaccine.[258] The basis for the idiosyncratic syndrome associated with the 1976 influenza vaccine is still not defined.

Future Considerations for Inactivated Virus Vaccine Development

Formaldehyde, heat, and oxidation have been shown to produce reactive carbonyl groups on protein Ags that promote CD4 T-cell responses with Th2 differentiation.[302] Chemical inactivation of poliovirus during vaccine manufacturing can result in modification of a major neutralization epitope. Therefore, inactivating infectivity of nonenveloped as well as enveloped viruses can adversely affect the antigenicity of the vaccine.[140] It has also been recognized that antigenic changes can occur during selection and amplification of influenza viruses in the allantoic cavity of embryonated eggs, the substrate used to produce virus for the inactivated vaccine.[392] The genetic basis for these mutations involves host cell selection of mutants that replicate more efficiently in eggs. These variants acquire a mutation in or near the receptor-binding pocket of the HA that increases their efficiency of replication in eggs but also alters antigenicity and immunogenicity. Nevertheless, influenza viruses used to prepare inactivated vaccine for humans are often grown in eggs, and such vaccines have been repeatedly shown to be protective. While this would not be a problem for influenza vaccines produced in mammalian cell culture, vaccine Ags may be modified or processed in ways that compromise antigenicity even in mammalian cell substrates. For example, poliovirus grown in tissue culture contains an uncleaved outer capsid protein VP1, which is usually cleaved in a natural infection by intestinal proteases. Although tissue culture grown poliovirus has limited ability to induce Abs that neutralize viruses containing a cleaved VP1,[393] both cleaved and uncleaved VP1 can induce protection from replication of subsequently administered live attenuated poliovirus vaccine.[366] Nevertheless, careful assessment of the substrate-dependent antigenicity and consequences of inactivation is warranted in the development of inactivated virus vaccines.

Viruslike Particle Vaccines

There are licensed VLP vaccines for two viral pathogens in the United States (HBV and HPV) and a vaccine for hepatitis E approved in China in 2017 (HEV 239). VLPs are produced as self-assembling capsid proteins in yeast or insect cells. The term VLP was coined in 1955 after studies of reconstitution of active tobacco mosaic virus.[150] For the purposes of this section, we will define VLP as self-assembling viral proteins that can mimic the particle structure of virions and present the quaternary structures of oligomeric viral surface proteins but do not package the virus genome. There are other self-assembling nonviral proteins that can be used to display vaccine Ags that are mentioned with nanoparticles under Delivery Vehicles section.

The initial version of the HBV vaccine was based on surface Ag purified from the blood of persons persistently infected with HBV. The Australian antigen was associated with the disease caused by HBV, and later the Dane particle was found to represent the Australia antigen. Interestingly,

electron microscopy revealed not only the Dane particle, which is a 42-nm structure representing the complete infectious virion, but also smaller particles and filaments. The 22-nm particle was found to be composed of just the hepatitis B surface antigen (HBsAg) and is noninfectious because it does not contain nucleic acid. This small particle, purified from human plasma, was the first licensed HBV vaccine. An effective second-generation HBsAg vaccine was developed using a recombinant plasmid to express the gene for HBsAg in yeast. Yeast-produced HBsAg self-assembles into uniform 22-nm particles. Although the 22-nm noninfectious particle is smaller in size and technically a defective genomeless subvirion particle, it is considered a VLP because it is made of the same capsid protein (HBsAg) as the full particle. Licensed HBV VLPs are formulated with alum and are now part of the routine pediatric vaccine schedule. This vaccine often is protective against HBV infection and hepatitis even in the absence of detectable Ab response. Immunization against HBV also significantly reduces the frequency of hepatoma and is regarded as the first anticancer vaccine.[72] The immunogenicity of HBV VLP in patients with renal failure, HIV-1 infection, or other immunocompromising conditions is diminished. New adjuvant approaches such as formulation with CpG (TLR9 agonist), MPL+alum, or MPL+QS21 are being explored to improve vaccine-induced immunity in those settings.[35,36,100] HBV VLP are now produced for human used in nine countries using yeast, bacteria, or mammalian cells for manufacturing, and other substrates including transgenic plants are being explored.[395]

HPV particles were first identified by electron microscopy in 1949. In 1983, the association between HPV infection and cervical dysplasia and malignancy was proven.[129] In 1991, it was shown that VLPs lacking genomes could be produced by expression of the L1 and L2[509] or just the L1[239] capsid proteins of papillomaviruses. L1 self-assembles into pentamers and each VLP is composed of 72 pentameric capsomeres. If present, one L2 protein is associated with the center of each L1 pentamer on the inner surface of the capsid. These key discoveries led to the development of candidate VLP vaccines. Importantly, the end points for the efficacy trial were chosen carefully. While prevention of cervical carcinoma was the goal, this target was not an acceptable end point because ablative treatment would be performed if premalignant lesions were detected. Therefore, cervical intraepithelial neoplasia (CIN) grade 2 or higher was used as the end point for vaccine efficacy trials, as a surrogate for cervical carcinoma. Prevention of persistent serotype-specific HPV infection was also a primary end point. Yeast-produced HPV 16 VLP formulated in alum and delivered parenterally was 100% effective in preventing CIN.[249] A three-dose regimen of a tetravalent L1-based VLP vaccine based on HPV 16 and 18 (most common cause of CIN) and HPV 6 and 11 (most common cause of anogenital warts) is also highly effective.[469] Subsequent studies have shown that HPV VLP immunization can prevent HPV-induced cervical lesions in women[382] and anogenital lesions in both women and men.[159,162] Three doses containing 30 μg of each of the four VLPs formulated with aluminum hydroxyphosphate sulfate (Gardasil) or 20 μg of each of two VLPs formulated with a mixture of aluminum hydroxide and 3-deacylated MPL (AS04) (Cervarix) are sufficient to achieve protective immunity that is durable for >5 years.[396] The protection has been correlated with type-specific neutralizing Ab. First-generation bivalent or quadrivalent vaccines immunized against two or four HPV types, but women were still exposed to additional high-risk HPVs that cause 30% of cervical cancer. The FDA approved a second-generation nonavalent HPV vaccine (Gardasil 9) that protects against an additional five high-risk HPVs (HPV types 31/33/45/52/58). The vaccine is highly immunogenic and may prevent ~20% more cervical cancers than the original vaccines.[415]

Advantages of Viruslike Particle Vaccines

Capsid proteins from many nonenveloped viruses and the nucleocapsid or matrix proteins together with surface proteins from some enveloped viruses can self-assemble into VLPs and provide a platform technology that may be applicable as vaccines for multiple virus families. VLPs offer several advantages as immunogens. First, the viral nucleic acid is not present in VLPs, so there is no concern that viral oncogenes or other pathogenic features of the live virus are present. Second, VLPs present conformational epitopes to the immune system in the same way as native infectious particles so that neutralizing Abs and other protective immune responses are induced efficiently. Some of these epitopes are formed by the juxtaposition of parts of two different capsid proteins. Third, many of the nonenveloped viruses from which immunogenic VLPs have been developed replicate poorly or not at all in tissue culture (eg, B19 parvovirus, papillomaviruses, and hepatitis E virus), thus precluding use of purified virus as immunogen. Fourth, because VLPs are noninfectious, chemical inactivation is not required. For this reason, VLPs might prove to be better immunogens than formalin-inactivated whole virus vaccines because the deleterious effects of formalin on structure and immunogenicity can be avoided. Fifth, the immune system responds better to a viral Ag presented as an assembled multimeric particle rather than as an individual monomeric protein. Sixth, the stability of VLPs from nonenveloped viruses may simplify vaccine storage and distribution and make successful oral delivery feasible in some cases.[332]

Disadvantages of Viruslike Particle Vaccines

The major disadvantage of VLP vaccines is related to the cost and complexity of manufacture, although costs should become lower with advances and expansion of the options for cell substrates. The biological disadvantage of VLPs compared to replication-competent viruses or vectors is that there is not ongoing production of Ag following vaccination. However, these particles tend to be highly antigenic and stable, and immunogenicity can be enhanced with adjuvants.

Future Considerations for Viruslike Particle Vaccine Development

VLPs have been engineered successfully for viruses belonging to a wide range of virus families, including both nonenveloped and enveloped viruses. Because of the advantages listed above and track record of success, they should be considered as a vaccine platform technology for other virus families when possible. VLPs have been synthesized in many expression systems as diverse as bacteria, yeast, plants, insect cells, and mammalian cells.[335] VLP-based vaccine candidates are being developed for prevention of many viruses,[450] including hepatitis B,[436] Ebola virus,[460] SARS-CoV-2,[500] the flaviviruses dengue and Zika, enterovirus A71, HIV, and influenza, among others.[20]

Nonenveloped Viruslike Particles

The first characterization of synthetic VLPs followed the observation that picornavirus capsid proteins could self-assemble during the *in vitro* translation of viral RNA to yield 75S to 85S particles.[351] Later studies indicated that VLPs could form *in vitro* following translation of picornavirus viral RNA lacking most of the 5′ noncoding region.[87] Next, it was observed that expression of the open reading frames of capsid proteins of nonenveloped viruses by a baculovirus or vaccinia vector in appropriate eukaryotic cells resulted in generation of VLPs.[88,239,509] For picornaviruses, particle assembly requires expression of the entire open reading frame to permit the proteolytic processing of the structural proteins by nonstructural viral-encoded proteases. There are current vaccine development approaches being used for enterovirus 71 that involve

VLP technology.[85]

Human parvovirus B19 causes erythema infectiosum, acute and chronic red cell aplasia, and other illnesses. A vaccine candidate consisting of the human parvovirus B19 VP1 and VP2 capsid proteins that form VLPs is being developed.[24] Immunization of humans with this vaccine induces neutralizing Abs that achieve levels that are equivalent in magnitude and neutralizing activity to those seen following natural infection.[46] Antigenic VLPs can be readily produced for other human (bocavirus)[71] and veterinary parvoviruses,[395] suggesting that VLP-based vaccines may be an appropriate approach for pathogens for this viral family.

Norovirus, the prototypic member of the calicivirus family, also may be a good candidate target for a VLP vaccine. The virus is a 28- to 37-nm nonenveloped particle that is formed by a single major capsid protein (VP1). VP1 is a protein with two domains connected by a short linker. The P domain forms the outer surface of the particle, and the S domain forms an inner shell. The P portion of VP1 can self-assemble 24 protomers to form a 20-nm particle that is immunogenic.[447]

Enveloped Viruslike Particles

Expression of the nucleoprotein or matrix proteins of some enveloped viruses has also resulted in the production of VLP or virus pseudoparticles. This finding was noted first for the Gag protein of HIV when it was expressed in recombinant vaccinia-infected cells.[192] Production of purified HIV pseudovirions is being developed as a vaccine strategy,[137] and the construction of vectors that are competent to produce pseudovirions *in vivo* may improve immunogenicity beyond that of the simple expression of the isolated gene products.[77,197] VLPs have now been produced for several respiratory viral pathogens. Expression of M1, M2, NA, and HA can form influenza VLPs,[259] but HA is sufficient in the presence of NA.[76] These are potent immunogens that can be constructed to express Ags that can confer broad cross-reactivity[434] or TLR agonists that can provide adjuvant properties.[473] SARS-CoV pseudoparticles are formed by transfection of 293 cells with the M (membrane) and N (nucleocapsid) genes.[213] The M protein of Newcastle disease virus (NDV) is sufficient to produce VLPs[353] that can support the incorporation and display of RSV F and G and is immunogenic and protective in animal models.[290] The NDV VLPs appear to be a stable platform for presentation of viral glycoproteins from other viruses as well.[307] Alphaviruses and flaviviruses are also potential viral targets for VLP vaccines. For example, West Nile virus pseudoparticles are formed by coexpression of prM and E.[199] Chikungunya virus VLPs were produced that protected rhesus macaques from lethal challenge.[3]

Novel chimeric particles in which viral Ags are built onto the hepatitis B core VLP[45] are immunogenic in mice. Relatively large structures can be mounted on the recombinant VLPs if inserted covalently into the outer loops or attached through chemical conjugation in the right place and orientation to allow access to the vaccine Ag being displayed. Other chimeric VLPs can be constructed by inserting candidate vaccine Ags into the variable loops that face outward including norovirus P particles,[446] chikungunya virus,[3] or others and could be selected based on the desired particle size and Ag valency (Fig. 15.5).

FIGURE 15.5 **Variations on VLP particle size and structure**. VLPs assume a wide range of sizes and are shown here as images derived from cryogenic electron microscopy. Hepatitis B (HBV),[126] hepatitis E (HEV),[497] bovine papillomavirus (PV) representative of other PVs,[488] and chikungunya (CHIKV)[3] are shown by order of increasing size.

Other Vaccine Approaches

Molecular biology has been an important stimulus for new approaches to make live attenuated virus vaccines and has also provided avenues for production of subunit vaccines and gene delivery vehicles that express viral Ags, especially mRNA (e-Fig. 15.6). MAb and recombinant DNA and mRNA technology are now used routinely to produce large quantities of purified viral Ags for use in immunoprophylaxis. MAb immunoaffinity chromatography or lectin chromatography facilitates purification of viral Ags from infected cells, while recombinant DNA technology makes it possible to express viral proteins in eukaryotic cells of yeast, fungal, insect, or mammalian origin. The use of these techniques has yielded candidate vaccines encompassing a large array of viral proteins that can induce protective Abs in experimental animals. Immunization by gene delivery is emerging as an important way to mimic certain aspects of live virus infection and can be accomplished by a growing list of delivery methods.

Vaccination With Proteins or Peptides

There are several theoretical advantages to immunizing with selected viral Ags instead of using whole virus particles. Production of recombinant viral proteins or synthetic peptides diminishes the risk of contamination by unrecognized pathogens. In addition, the preparation of vaccines free of nucleic acid eliminates theoretical concerns about recombination or integration of viral nucleic acid. Immunizing with individual Ags or epitopes from the virus might also reduce antigenic competition and focus the response on the most relevant protective antigenic sites.

Production of Viral Proteins in Eukaryotic Cells

Before expression of viral proteins in eukaryotic cells can be used as a method for vaccine production, several obstacles must be overcome. First, it is essential to identify the correct immunogen. Second, it is necessary to produce protein in a highly efficient manner to make this approach economically feasible. Mechanisms to enhance expression of viral proteins that have been pursued focus primarily on identification of strong promoters of gene expression and development of techniques for efficient gene amplification. Third, viral proteins must be separated effectively from host cell protein, DNA, and endogenous adventitious agents. Lastly, the proteins must be produced with the desired structure and activity. This latter requirement is of special concern since immortalized mammalian cell lines are ideal for producing viral proteins because posttranslational modifications are more likely to be authentic than when these proteins are produced from prokaryotic hosts. Host cell DNA must be greatly reduced in the final vaccine (FDA guidance is <10 ng per dose) to reduce the theoretical potential for oncogenic events. Additionally, removal and inactivation of endogenous viruses must be performed for products made from continuous rodent and other mammalian cell lines. Recombinant DNA techniques to promote secretion of viral glycoproteins into the medium have been developed to facilitate effective separation of viral proteins from host cell proteins. Fourth, the procedures used to produce and purify viral protein must be gentle enough to maintain the protein in its native state thereby preserving conformationally dependent neutralization epitopes needed to induce protective Abs. Finally, because purified monomeric proteins are typically weak immunogens, they need to be formulated with adjuvants

or produced as oligomers or particles to enhance their immunogenicity. The following discussion of these approaches is not intended to be exhaustive in scope but illustrative of current strategies in use or in development.

Recombinant DNA techniques are commonly used to express viral proteins in yeast, insect, or mammalian cells. The usefulness of yeast expression to produce the HBV vaccine has been described above, and yeast is also used to produce HPV VLPs.[271] Some viral proteins such as the rabies G glycoprotein are more difficult to produce in yeast.[408]

A major advance in viral Ag production has been the development of baculovirus vectors to express foreign genes to high levels in insect cells under control of a strong baculovirus promoter such as the polyhedrin promoter.[274] Protein can be produced in large quantity (1 mg/10^6 cells) using this system. Viral glycoproteins also can be produced in this system. For example, baculovirus-produced influenza hemagglutinins can be produced efficiently and have equivalent or superior immunogenicity to the traditional trivalent split product in adults.[30] Influenza HA–based protein vaccines produced in insect cell culture have been approved in the United States since 2013 (Flublok or Flublok Quadrivalent). The baculovirus system also can be used to express more complicated viral immunogens that require polyprotein processing to generate native immunogenic surface proteins. An example of this is expression of the entire structural protein region (C-E3-E2-6K-E1) of an alphavirus to generate authentic, processed viral proteins capable of inducing an efficient neutralizing Ab response in experimental animals.[209]

Mammalian cells also have been used to produce viral proteins that can be incorporated in a subunit vaccine. Mammalian cell cultures represent an optimal system to produce viral proteins because protein folding, transport, and processing closely approximate those that occur in the infected host. Initial approaches were to purify viral proteins from infected cells or from cells transduced with a recombinant virus expression system engineered for high level of expression of the foreign viral Ag[153,154] or by transient transfection with DNA. Cell lines have been developed that can stably produce recombinant proteins or Abs, can be modified for large-scale production, and have achieved regulatory approval for clinical products.

Chinese hamster ovary (CHO) cells have long been a preferred cell line to produce protein-based therapeutics.[459] These cells have been used to produce viral glycoproteins from HIV and HSV that have been advanced to efficacy trials[147,388,438] and can be modified to improve the manufacturing efficiency and capacity even further. Immortalized human cell lines derived from healthy human embryonic material have also emerged as producer cell lines for protein production such as 293 and PER.C6. Both lines have been used extensively to produce gene delivery vectors, have fully documented passage histories, and have had no adventitious agents or oncogenic or teratogenic potential identified to date. PER.C6 was derived from embryonic retina cells and immortalized with the adenovirus E1 gene. 293-ORF6 has a similar development history and contains the adenovirus open reading frame 6 from E4 as well as the E1 gene.[59] These fully human cell lines may provide advantages for producing virus glycoproteins that authentically replicate the structures present in human infection, can be adapted to different growth conditions, and can stably produce high levels of recombinant protein without gene amplification.[223] In general, identifying immortalized human cell line platforms to produce proteins, VLPs, whole virus, or vaccine vectors will be preferable to using primary cell lines that have to be revalidated repeatedly or using less efficient production methods that require transient infection or transfection.

Production of Viral Proteins in Prokaryotic Cells

Production of immunogenic viral proteins in prokaryotic cells is difficult and requires extensive re-engineering of the gene to achieve immunogenic structures because of differences in protein processing and in microenvironment. However, there have been some notable successes, particularly in the production of VLPs. A portion of the structural protein from hepatitis E ORF2 produced in *Escherichia coli* self-assembles into particles and was shown to be efficacious in a 100 000-person study.[511]

Production of viral proteins in plant cells

Plants have been modified genetically to express virus proteins[68,444] and VLPs[110] some of which have advanced to clinical trials. Plants can be engineered with selected mammalian glycosidases to provide more authentic posttranslational modifications.[440] The major advantage of this approach is the potential for inexpensive and widely distributed manufacturing capacity.

Protein Purification

Immunoaffinity chromatography, lectin chromatography, and physical separation techniques have been used to purify a variety of viral glycoproteins that have induced partial to complete protection in animals. When directly compared in animals, protection induced by immunization is greatest when the purified viral glycoprotein is in its native conformation.[463] In the case of RSV and EBV, purified glycoprotein vaccines induced Abs that were able to bind to glycoprotein in enzyme-linked immunosorbent assays (ELISA), but these Abs had a very low level of neutralizing activity.[323,487] In contrast to purified protein immunogens, RSV glycoproteins expressed by a vaccinia virus RSV F or G recombinant induced a titer of neutralizing Abs comparable to that developed by animals infected with the virus.[96] Observations of this type underscore the potential importance of quaternary structures and presentation of proteins in the context of membranes and organized on particles to achieve functional Ab responses.

Synthetic Peptides as Immunogens

During the early 1970s, the use of synthetic peptides as immunogens was studied using a synthetic icosapeptide representing the immunodominant domain of the outer coat protein of bacteriophage MS-2.[257] A 56 aa peptide from the stem region of the influenza hemagglutinin can induce Abs to a conserved epitope that has some cross-neutralizing activity.[477] However, the immunity induced by peptides is usually less than that achieved when full-length protein, inactivated whole virus, or live virus was used as immunogen.

The fact that CD8 T-cell epitopes are short peptides of 8-10 amino acids in length presented in the context of the MHC class I heterodimer suggested that it might be possible to produce peptide vaccines to induce CTL-mediated immunity.[132] CTL induction by peptide vaccines has been achieved in several experimental systems. For example, a peptide, in adjuvant, induced CTLs and protected mice against Sendai virus infection.[232] An epitope-based vaccine candidate termed Multimeric-001 (M-001) is a single recombinant protein that consists of three repetitions of nine conserved linear epitopes from HA (four B-cell and one CD4 T-cell epitopes), NP (two CD8 T-cell and one CD4 T-cell

epitopes), and M1 (one peptide containing both B-cell and CD8 T-cell epitopes), which were derived from influenza A and B strains expressed in *E coli*.[34] Preclinical studies in animals showed protection, but a phase 3 clinical trial completed in October 2020 failed to meet the trial's primary and secondary efficacy end points. Thus far, peptide vaccines have not achieved significant immunogenicity when used in immunologically naive humans unless formulated in preparations with strong adjuvants that are associated with unacceptable reactogenicity. Also, in general, the reductionist approach to vaccine Ags has not been successful when tested in humans. In general, it is better to use a larger antigenic content in vaccines for viruses, so each individual host can select the most relevant and effective epitopes for themselves. This principle is also consistent with the concept that vaccination has its largest impact on public health at the population level. Developing tailored, personalized vaccination approaches may be in the distant future for preventive antiviral vaccines, and this type of approach is currently being explored for therapeutic vaccines against neoplasms and some chronic virus infections. Indeed, immunization with mixtures of long peptides derived from HPV E6 and E7 oncoproteins formulated in IFA has led to remission of HPV16-induced vulvar intraepithelial neoplasia.[233]

Vaccination by Gene Delivery

The age of molecular biology has made it possible to build expression vectors that can serve as a vehicle for gene delivery of vaccine Ags. The development of this technology is attractive because it combines the immunogenicity advantages of live vaccines and the safety advantages of nonliving vaccines. Genes can be modified to be nonfunctional and harmless to the host, improve expression, and yet retain the antigenicity of the expressed protein. Using the host cell for transient protein production provides the opportunity for expression of more native structures than are possible with *in vitro* protein production including glycosylation patterns that may influence antigenicity. Genes can be produced synthetically so that no component of the original virus pathogen is needed in the manufacturing process to avoid the potential complications of infection with live viruses or adventitious agents. Selected immunogenic components of the pathogens can be expressed and combined with molecular adjuvants that enhance and guide the composition of immune responses. In addition, the mechanisms that viruses use to evade or alter immune responses can be removed, or genes can be modified (eg, by adding ubiquitination sites) to improve processing and Ag presentation.[433] Therefore, it may be possible to improve on the immunity afforded by natural infection by using vector-based expression of viral Ags. New delivery approaches, including vector technology, provide the opportunity to combine Ags from multiple strains or different pathogens to potentially allow broadening of vaccine-induced immunity while simplifying vaccination regimens. They can be categorized by their functional properties (e-Fig. 15.7) and other features that may influence vaccine strategies for a particular virus (e-Table 15.2).

e-FIGURE 15.7 **Functional characteristics of vectors used for gene expression.** Vectors used for gene delivery have several different properties that may have advantages or disadvantages for a given vaccine development project. For example, replication competent vectors may have advantages if the major goal is direct induction of mucosal immunity. If the vaccine Ag being expressed is large or has a complicated secondary RNA structure, some vectors may be preferred based on insert capacity or whether nuclear or cytoplasmic transcription utilized.

e-TABLE 15.2 Considerations for vaccine vector selection

Nucleic Acid Vaccines

The concept of using mRNA or DNA to deliver vaccine Ags has many attractive features, including ease and flexibility of construction, scalable manufacturing capacity, stability, production of vaccine Ag in host cells, transient expression, no induction of antivector immunity, and induction of a balanced CD4 and CD8 T-cell response in addition to an Ab response. Gene-based immunization promotes host cell synthesis and expression of the vaccine Ag and physiologic posttranslational processing and folding in the cell cytoplasm. Immunization with mRNA or DNA has some theoretical advantages over immunization with purified viral Ags. The most important advantage is that vaccine Ags such as viral glycoproteins can be expressed on the surface of transfected cells and are presented to the immune system in a native state. During the processes of purification of viral proteins, purification and assembly of VLPs, or inactivation of viruses with chemicals, epitopes on viral proteins can be altered compromising immunogenicity. Gene-based immunization, which circumvents these problems, more closely resembles immunization with a live virus vaccine than with an inactivated viral Ag. Viral Ags encoded by nucleic acids, whether entire proteins or minimal CTL epitopes, are presented efficiently in the context of MHC class I molecules and are able to induce CD4 T cells and CTLs.[70,261,282,504] In this way, it is possible to induce a balanced immune response more like immunity induced by natural infection than to immunity induced by administration of preformed viral Ags. A second advantage of immunization with nucleic acids is their ability to transfect cells without interference by antiviral or antivector Abs. Live attenuated vaccine virus, such as that present in the measles virus vaccine, is effectively rendered noninfectious by Abs. Likewise, Abs to gene delivery vectors, such as adenovirus serotype 5 (Ad5), can diminish the immunogenicity of this approach, while mRNA or DNA immunization is not affected by preexisting Abs.

mRNA Vaccines

Lipid nanoparticle (LNP)-encapsulated nucleoside-modified mRNAs have recently deployed as a robust vaccine platform, exhibiting exceptional potency and rapid developability. The development of mRNA vaccines during COVID-19 was astoundingly rapid but was based on decades of fundamental research. RNA was used for synthetic gene expression as early as the 1970s, but typically induced cell programs that inhibited expression after triggering of TLRs. Karikó and Weissman determined that the use of modified RNA (such as substituting uridine with the analogue pseudouridine) reduced cellular recognition of the foreign RNA and enhanced protein expression. Delivery systems also needed to be developed to protect RNA (which is susceptible to rapid degradation in biological matrices) and to deliver the RNA into target cells. Investigators discovered various combinations of lipids that could cover synthetic mRNAs forming lipid nanoparticles. Particular lipids in these mixes also have adjuvanting effects, which allowed development of prototype viral vaccines starting with influenza, and later Ebola and other viruses. By early 2020, the company Moderna had advanced nine mRNA candidates for infectious diseases into the clinic. Soon thereafter, the COVID-19 pandemic occurred and SARS-CoV-2 vaccines to prevent COVID-19 were rapidly developed by Moderna and Pfizer-BioNTech using nucleoside-modified mRNAs encoding prefusion stabilized spike protein and incorporated into LNP vaccines (Fig. 15.6). The vaccines are highly effective and were rapidly approved by U.S. and European regulatory bodies in 2021. These vaccines are remarkably immunogenic, protective, and safe,[18,378] although the durability of the antibody response is short, requiring multiple boosters to sustain maximal serum antibody levels. Many other LNP-mRNA vaccine candidates are now being studied, and some have already been shown to be effective against diverse pathogens in preclinical studies. Several clinical studies are underway to prevent viral diseases including studies of mRNA vaccines for human CMV (ClinicalTrials.gov identifier NCT04232280) or human RSV (ClinicalTrials.gov identifier NCT04528719). Interestingly, antibodies for viruses also can be delivered by mRNA LNP preparations; the first soluble protein delivered to humans by mRNA was a human mAb to chikungunya virus tested in a phase 1 trial in 2019.[17]

FIGURE 15.6 **Prototype of a COVID-19 mRNA lipid nanoparticle vaccine**. The figure represents the constituents of the Moderna Spikevax vaccine. The mRNA is a nucleoside-modified mRNA encoding the spike of SARS-CoV-2. All uridines are substituted since modified nucleosides in the mRNA make it less inflammatory and more translatable. The mRNA is encapsulated in lipid nanoparticles comprising an optimized lipid mix containing polyethylene glycol, 2000 dimyristoyl glycerol, 1,2-distearoyl-sn-glycero-3-phosphocholine, cholesterol, and SM-102 (proprietary) that stabilize the mRNA and facilitate its entry into cells.

DNA Vaccines

It was originally found to be possible to express transgenes by direct intramuscular or intradermal inoculation of plasmid DNA in the early 1990s.[448,489] The first demonstration of DNA vaccine protection against a virus was in mice challenged with influenza.[305] Subsequently, immunization with DNA that encoded viral Ags, including Ags that can form VLPs, from a wide range of viruses has induced functional (ie, neutralizing or syncytium-inhibiting) Abs and/or resistance to viral infection.[122] For these reasons, DNA vaccination can elicit both CD4 and $CD8^+$ T lymphocyte responses with a variety of immunogens in animal models. DNA immunization in humans has induced significant neutralizing Ab[261,282] and T-cell responses.[176] The delivery process is partially improved by using newer delivery devices and new approaches incorporating sonoporation with sound waves or electroporation, which offer the potential for improved potency of DNA vaccines.[416] DNA vaccines have not been approved, however, for human use. One veterinary DNA vaccine was licensed in 2005 by the USDA, an equine vaccine for West Nile virus. The major factor limiting the immunogenicity of DNA vaccines is inefficient transfection of cells in a way that achieves transport of genes into the cell nucleus. DNA is a large, charged molecule that is only active in the cell nucleus.

Replication-Competent Vectors

The molecular technologies that have made gene delivery possible in general have been applied to the development of stable attenuated virus vaccines, some of which have been used to construct viable recombinants that express the surface proteins of other viruses, thus blurring the distinction between live attenuated viruses and vaccine vectors. Chimeric or live recombinant virus vectors can be designed to deliver the foreign gene from a virulent pathogen in the context of a nonpathogenic expression system, thereby improving safety. The vector design can also consider manufacturing issues to improve overall production efficiency. The use of a viral vector for immunization has the advantage that the foreign viral Ags are expressed naturally in the context of an infected cell thereby inducing cellular as well as humoral immune responses. Vector selection and design can focus vaccine Ag expression to a particular target cell or immunological compartment to tailor the organization of an immune response to a particular pathogen. Delivering the vaccine Ag in a complex structure has the benefit of broadly engaging both the innate and adaptive immune responses to better simulate the immunity to natural infection. Replication-competent vectors have additional immunogenicity advantages over replication-defective vectors because more prolonged Ag expression and more diverse stimulation of TLR pathways often translates into better Ab production and more durable immune responses.

Chimeric live virus reassortant and recombinant vaccines

Two major platforms have emerged to create chimeric viruses. One is based on reassortment of genes that occurs in segmented RNA viruses and is used in two currently licensed vaccines. The other utilizes reverse genetic systems developed to make molecular clones of viruses with nonsegmented RNA genomes. Live attenuated chimeric virus vaccines are distinguished from live viral vectors because the viral vaccine Ag being expressed is derived from the same virus family and replaces the homologous protein from the virus that supplies the coding and noncoding regions constituting the replication competent particle.

The rhesus and bovine rotavirus vaccines are examples of live attenuated chimeric viruses produced by reassortment. The chimeric viruses contain the attenuating background of the rhesus or bovine rotavirus plus the gene for a human rotavirus VP7 surface protein. Similarly, the live attenuated influenza virus vaccines are chimeric viruses, which can be antigenically updated by replacement of the HA and NA genes of the attenuated donor virus with those of a new epidemic or pandemic virus. This process was utilized in the response to the 2009 H1N1 pandemic. While the replication of A/California/7/2009 H1N1-like isolates in eggs was relatively low, the chimeric live virus produced as FluMist had outstanding growth properties that facilitated production.

Construction of recombinant live attenuated chimeric virus vaccines by gene replacement in molecular clones has mostly used flaviviruses or paramyxovirus backgrounds. For flaviviruses, a live attenuated virus vaccine candidate for JEV has been made by the replacement of genes encoding the membrane precursor (prM) and envelope (E) proteins of the attenuated yellow fever vaccine virus (YFV) with those from an attenuated strain of JEV. The resulting JEV-YFV antigenic chimeric recombinant vaccine candidate was attenuated and immunogenic *in vivo*.[185] Both components of this chimeric virus came from attenuated viruses. It has now been evaluated in advanced clinical trials[303] and is being submitted for registration.[188] Chimeric recombinant candidate vaccines have been made between a naturally attenuated tick-borne flavivirus (Langat virus) and a wild-type mosquito-borne dengue type 4 virus (DENV-4), and the resulting recombinant was found to be significantly more attenuated than its tick-borne parent virus for mice.[371] This is an example of an attenuating effect that stems from partial incompatibility between the two components of the chimeric virus. A third strategy is being pursued to produce a tetravalent dengue virus vaccine in which a DENV-4 background containing an attenuating deletion mutation in the 3′ noncoding region is used to construct antigenic chimeric viruses containing the surface proteins of other dengue serotypes.[294] Alternatively, the 3′ attenuating mutation is reproduced in the other serotypes. Chimeric flavivirus constructs appear to be a stable and effective vaccine platform for these types of viruses. A tetravalent, live attenuated dengue vaccine based on the attenuated DENV-4 background is immunogenic in humans and has been evaluated in advanced clinical trials.[188,324] A dengue vaccine containing a chimeric YFV 17D strain expressing prM and E from each of the four dengue serotypes (designated CYD-TDV; Dengvaxia) has been approved for use in persons previously infected with dengue. This vaccine is only intended for use in previously infected individuals and is partially effective in preventing infection. Inadvertent vaccination of dengue-naive individuals, however, may lead to a higher risk of severe disease during subsequent natural infection.

A chimeric virus containing the HN and F surface proteins of parainfluenza virus type 1 substituted for those of an attenuated PIV3 virus has been constructed and shown to be attenuated and protective in experimental animals.[449] Both bovine and human PIV3 chimeras are being pursued to make vaccines against several other paramyxoviruses including PIV1, -2, and -3; RSV; metapneumovirus; and measles virus. Both human and bovine PIV3 are very amenable to use as a background for chimeric paramyxovirus vaccines, but other paramyxoviruses are also being developed as vaccine vectors including Sendai virus, Newcastle disease virus, canine distemper virus, and measles virus.[214] Paramyxoviruses have also been constructed as vectors to deliver vaccine Ags from unrelated viruses such as HIV or Ebola.[118]

Poxvirus vectors

Vaccinia virus is the most used recombinant vector to date.[311] Poxviruses have large genomes into which a variety of foreign viral genes can be inserted and expressed without seriously compromising the capacity of vaccinia virus to replicate. Recombinant vaccinia viruses expressing the surface protein(s) of many viruses have been constructed and shown to be protective in experimental animals. Polyvalent live vaccinia virus recombinants have been used to successfully immunize experimental animals, even in the presence of immunity to one of the foreign viral Ags,[144] and vaccinia-rabies virus recombinants are being used to control rabies in foxes[358] (e-Table 15.3). Recombinant vaccinia has been evaluated in clinical trials,[172-174] but they are not sufficiently attenuated for use in immunocompromised hosts[139,184,386] and are complicated by other idiosyncratic side effects like myopericarditis.[14] However, new versions of attenuated replication-competent poxvirus vectors like the Tiantan strain of vaccinia have been constructed as candidate HIV vaccines and are currently being evaluated in clinical trials in China.[113]

e-Table 15.3 Licensed veterinary viral vaccines

Herpesvirus vectors

Herpesvirus vectors have been developed using herpes simplex virus type 1 (HSV-1), EBV, CMV, and some animal alphaherpesviruses. The primary advantages of this vector system are the capacity to package up to 100 kb of foreign DNA and the potential for long-term gene expression from latent episomal DNA that can be present in both neuronal and nonneuronal cells.[57] Herpesviruses have tropism for a wide variety of cells and are efficient at cell binding and attachment even at low particle to cell ratios. Vectors can also be pseudotyped to help target delivery into selected host cells. They can be given mucosally as well as parenterally and can be engineered to eliminate immune evasion genes like the HSV-1 ICP47 that interferes with the TAP to diminish MHC class I presentation. Live herpesvirus vectors can be attenuated to avoid vector-mediated disease and can also be made replication defective (see below). Because HSV is ubiquitous, a potential disadvantage for this approach is that preexisting Ab to the vector may diminish immune responses to the transgene product. In a murine model, it was shown that preexisting HSV immunity did not alter the Ab or proliferative responses to recombinant protein encoded by the HSV vector.[51] Using animal herpesvirus vectors may be an alternative approach to circumvent the potential problem of preexisting immunity to the vector, although replication is severely restricted when species barriers are crossed.[457] There is a licensed veterinary vaccine for Marek disease in chickens based on this technology (e-Table 15.3), and this technology has also been applied in HIV vaccine development. Initially, it was shown that replication-competent HSV vectors expressing Env and Nef showed partial protection against challenge with SIVmac239.[325] Subsequently, rhesus CMV vectors were constructed expressing Env, Gag, Pol, and Rev/Nef/Tat. A clinical trial is in progress using a human CMV vector to express HIV T-cell epitopes (Clinicaltrials.gov NCT04725877). Animals immunized with either recombinant CMV vectors alone or primed with CMV and boosted with recombinant adenovirus vectors had significant protection from an SIVmac239 mucosal challenge. The correlate of protection is thought to be persistence of tissue-resident effector memory CD8 T cells, sustained by the persistence of the vector.[200]

Rhabdovirus vectors

Vesicular stomatitis virus (VSV) has been engineered to serve as a vector for recombinant gene delivery. Recombinant VSV can be produced at high titer, is relatively stable, has broad tropism, and can be given mucosally.[236] VSV is a rhabdovirus and is known to have neurovirulence. Therefore, extensive work has been done to attenuate the parent vector by altering the gene order and by truncating the cytoplasmic tail of the major surface glycoprotein.[380] Gene delivery and subsequent immune responses to the recombinant gene product are potent.[193] A vaccine based on VSV that was genetically modified to express the surface glycoprotein of Zaire Ebola virus (designated VSV-EBOV or rVSV-ZEBOV) was approved in the European Union and in the United States in 2019. Rhesus macaques immunized by a VSV vector expressing envelope and gag have been protected from SHIV challenge.[398] Replication-defective VSV vectors have also been developed (see below).

Live adenovirus vectors

The live adenovirus vaccine for serotypes 4 and 7 is a licensed and highly effective vaccine that is administered mucosally. Therefore, it has ideal properties for a gene-delivery vehicle and has been developed as a candidate HIV-1 vaccine.[273,362] The advantages are the track record of the vector for both safety and efficacy, and the potential for delivery in capsules to the lower intestinal tract.[186,445]

Picornavirus vectors

Poliovirus vectors have been developed that can express recombinant foreign proteins that are excised by the virus-encoded protease after translation of the recombinant polyprotein.[11] The major advantages of this approach are the long history of successful vaccination with the vector, and the capacity for oral delivery and induction of mucosal immunity. The major disadvantage of this system is the relatively small packaging capacity (~200 aa) that requires a library of recombinant viruses in the vaccine to deliver large Ags. Enteroviruses and rhinovirus vectors have been developed using similar technology and to target different mucosal inductive sites, and new innovations like removing part of the capsid gene and can provide a larger coding region for the vaccine Ag.[120,315]

Bacterial vectors

Bacterial vectors have also been used to deliver viral Ags. Recombinant BCG has received the most attention. Some advantages of BCG as a vector include the following: (1) its safety record after administration to millions of persons especially in low- and middle-income countries (although BCG can cause local or lymph node complications [BCGitis] or disseminated infection [BCGosis] in immunocompromised recipients); (2) its persistence, and therefore prolonged Ag production that may provide a more durable immune response; (3) its potential for mucosal administration; (4) its innate chemical composition that provides an adjuvant effect through TLR-1, TLR-3, TLR-4, and possibly other pattern recognition receptors; and (5) immunity against tuberculosis could be a by-product of immune responses to the vector. Advances in mycobacterial genetics have made construction of recombinant vectors feasible,[412] and because the large capacity for accepting foreign genes, coexpression of cytokines and other immunomodulators is possible.[6,326] In addition, discoveries about how mycobacteria inhibit apoptosis of macrophages to delay immune-mediated clearance have led to the development of attenuated mycobacteria for vaccination against tuberculosis. New promoters, other vector design modifications, and combining with other modalities may further enhance immune responses to the foreign

gene products.[397] Other bacterial vectors that have been considered for delivering viral Ags include lactobacilli, *Salmonella*, *Shigella*, and *Listeria*. The *Salmonella* and *Shigella* vectors have also been designed to deliver the gene-based vaccine Ag by plasmid and thereby combine mucosal delivery and adjuvanticity with authentic glycosylation of the gene-product.[148] This concept has been tested in early phase clinical trials to express an HIV-1 envelope protein, but there was poor immunogenicity.

Replication-Defective Vectors

Replication-defective virus vectors have had genes deleted that are essential for replication *in vitro* or *in vivo*. Replication-defective viruses can replicate to high titer in cell lines engineered to express the missing protein that would be encoded by the deleted gene. The viruses produced in such complementing cells can attach and deliver their genetic payload to cells *in vivo* but are unable to replicate. Such vectors undergo an abortive infection *in vivo* but express the foreign protein during this abortive infection and can thereby induce both the humoral and cellular arms of the immune response. Nonreplicating vectors do not retain the virulence of the original agent from which they were derived and would be expected to be safe even in hosts with compromised immune systems, should they encounter the vector. Many vectors do retain the antigenicity of structural proteins from the original agent. This raises two issues. The first is reactogenicity. Many vectors can trigger innate immune responses that cause local and systemic reactions in the vaccinee. This is an advantage from the perspective of adjuvanticity but can be limiting at higher doses of the vector. The second is preexisting immunity to the vector. If the vector is derived from an agent to which the host has had prior exposure, the preexisting Ab can dampen the immunogenicity of the delivered vaccine Ag. Vector-based vaccines are of value when the vaccine target is a virus for which live attenuated or whole-inactivated virus vaccines are not considered for safety or other reasons. Vectors have added value when the vaccine strategy is focused on inducing CD8+ T-cell responses because they deliver the vaccine Ag to the cytoplasm allowing access to the MHC class I processing pathway for Ag presentation. Because this characterizes the current strategies for HIV vaccine development, most vector-based vaccine strategies have been evaluated for the induction of protective immunity against lentiviral infection. Therefore, antiviral vaccine vectors will be discussed primarily in the context of HIV vaccine development, even though they are also being developed for many other antiviral vaccine programs.

The regulatory approval pathway for vector-based gene delivery has involved scrutiny around issues such as integration, recombination, reversion to replication competence, and reactogenicity. The origins of many vaccine vectors are from the field of gene therapy, and some of the clinical events associated gene therapy trials greatly affected the evaluation of vaccine vectors. For example, the 1999, death of a teenager in a gene therapy trial at the University of Pennsylvania prompted extensive reviews of safety data from both human and animal studies of vector-based gene delivery.[16] This incident compels a cautious approach to dose and route evaluation of any antigenic substance, but particularly vector-based products. Another gene therapy incident involved a retrovirus vector used to successfully treat children with X-linked Severe Combined Immunodeficiency. After 3 years, some of the children developed leukemia associated with the integration of vector DNA into the LMO-2 protooncogene locus.[190,191]

Adenovirus vectors

Replication-defective recombinant adenoviruses (rAd) exhibit characteristics that make them ideal vaccine vector candidates. Adenovirus serotype 26 (Ad26) is a low-prevalence naturally occurring human adenovirus known to cause common cold–like symptoms, which was manipulated by gene deletion to develop a replication-defective vector. Johnson & Johnson (J&J) Janssen has used this vector to develop two approved viral vaccines. A two-dose regimen of this vector expressing Zaire Ebola virus glycoprotein (Ad26.ZEBOV) followed by a nonreplicating, recombinant, MVA vector–based vaccine, encoding glycoproteins from Zaire Ebola virus, Sudan virus, and Marburg virus, and nucleoprotein from the Tai Forest virus was approved for medical use in the European Union in July 2020. The company also developed a vector that encodes a full-length and stabilized SARS-CoV-2 spike protein that obtained EUA from the U.S. FDA in 2021. Substantial resources are being invested to develop safe and immunogenic rAd vaccines for other infectious agents including HIV, plague, anthrax, influenza, tuberculosis, and malaria.[27] These vectors possess desirable qualities for use in vaccination strategies, including efficient transduction of host cells, limited duration of gene expression, vector nonpersistence, the ability to rapidly induce both humoral and cell-mediated immunity, induction of innate immune responses that may provide an adjuvant effect, relatively high stability, and ability to scale up production for manufacturing purposes.[27] Most rAd vaccine candidates are replication-defective due to a deletion of the E1 region. Some vectors have additional deletions of E3 and E4 to increase capacity for gene insertion and to further reduce the chance of recombination in the packaging cell line to form replication-competent adenovirus. Deletion of E4 also prevents transcription of adenoviral genes encoding structural proteins to reduce antigenic competition with the vaccine Ag. Complementary human embryonic cell lines such as PER.C6[275] or 293-ORF6[52] are used to package the deficient rAd *in vitro* and allow large-scale production. Preexisting immunity to a given adenovirus serotype has been shown in animal models and in human clinic trials to diminish the immunogenicity of the vaccine Ag. Ad5 immunity has a high prevalence, particularly in developing countries.[245] Although Ad5 has been commonly used in the initial studies, there are 51 human Ad serotypes, and many have now been engineered to express foreign genes. In addition, new vectors have been constructed from adenoviruses derived from chimpanzees and macaques that have favorable properties and will provide options to avoid preexisting vector-specific immunity. An alternative to using a rare serotype recombinant adenovirus vector is to construct chimeric vectors. For example, adenovirus vectors have been developed in which the shaft and knob portions of the fiber proteins are exchanged.[496] Another novel approach involved making hexon loop chimeras in which the neutralizing targets in hexon are exchanged for those of an uncommon adenovirus serotype.[443] A mucosal route of administration is another potential option for overcoming preexisting vector immunity, with the added advantage of better induction of immunity in the mucosal compartment. This may be particularly important for inducing immunity against viruses that primarily enter the host through a mucosal surface like HIV and influenza. Replication-defective rAd has been successfully given mucosally without adverse reactions,[464] even though one study in mice showed that intranasal administration could result in Ag expression in the olfactory bulb.[263] The effects of preexisting immunity to the vector can also be partially overcome by increasing the dose or by priming with a heterologous vector expressing the vaccine Ag of interest. Finally, one can avoid preexisting immunity by carefully selecting the target population. For example, children between 6 months and 2 years of age are Ad5 seronegative, which makes rAd5 vectors a viable choice for immunization of that age group.

The Step study was a phase IIb trial that evaluated E1-deleted rAd5 vectors expressing HIV-1 Gag, Pol, and Nef in individuals at high risk of HIV infection. It was stopped prematurely in 2007 because there was a higher frequency of HIV-1 infections among vaccinees than placebo recipients.[53] The increased rate of infection was associated with being uncircumcised and with preexisting Ad5 Ab. The biological basis for this outcome is not known. HIV-1 vaccine clinical trials utilizing rare serotype rAd vectors and rAd5 vectors (E1, E3, E4-deleted expressing HIV-1 Env, Gag, and Pol) in heterologous prime-boost combinations with DNA vaccines are still in progress.

Adeno-associated virus vectors

Adeno-associated virus (AAV) vectors are unique because the wild-type virus itself is replication-defective and requires the presence of wild-type adenovirus as a helper virus to propagate. The vector is very simple and contains the promoter, foreign gene, and polyadenylation sequence surrounded by two short noncoding inverted terminal repeats from AAV. No genes from the wild-type AAV are included in the vector. Particles are produced in a packaging cell line that provides the AAV structural proteins. The AAV capsid induces an immune response, and like other vectors, anti-AAV Ab can diminish immune responses to the foreign gene product expressed by recombinant AAV vectors. Using alternative capsid serotypes and incorporating immune modulators may overcome any limitations posed by preexisting AAV immunity or allow heterologous boosting and further amplification of immunogenicity induced by primary vaccination. Transduction of muscle cells and Ag-presenting cells is efficient, and expression can be prolonged. A single dose of recombinant AAV has been shown to induce both humoral and cellular immunity sufficient to control SIV infection in macaques.[222] AAV-transduced cells achieve persistent gene expression by delivering concatemeric circular episomes that remain transcriptionally active and there is no evidence for integration.[421] Long-term episomal gene expression has been uniquely demonstrated by delivering neutralizing Ab genes to muscle cells in rhesus macaques and achieving sufficient titers in serum to protect against SIV challenge.[221] It should be noted that dozens of AAV-based gene therapies are in late-stage clinical development to treat genetic diseases, and several have been approved starting in 2012.

Alphavirus vectors

Alphaviruses like Venezuelan equine encephalitis virus (VEE), Sindbis virus, and Semliki Forest virus (SFV) have been modified to produce three distinct types of immunogens. All take advantage of the alphavirus replicon that is a minimal genome with replication origins and packaging signals. The vectors include the alphavirus nonstructural genes including the replicase and the subgenomic promoter upstream of a foreign gene that replaces the genes encoding alphavirus structural proteins. The replicon itself can be injected as either plasmid DNA or as naked RNA. Alternatively, helper cell lines can be constructed that provide the structural gene products needed for packaging of replicons and particle assembly of nonpropagating vectors.[153] These replication-defective viral particles are extremely efficient in targeting Ag-presenting cells, particularly DCs, and because of the amplification of the RNA, achieve high-level expression of the foreign protein. They have induced significant humoral and cellular immune responses to a variety of viral proteins in animal models,[208,222,381] and a VEE vector expressing HIV Ags has advanced into clinical trials.[486] The immunogenicity of the VEE vector is in part due to its tropism for DCs. When minor changes are made in the envelope protein to diminish DC entry, the induction of immune response is compromised.[276] One unique property of VEE vector–induced immunity is that parenteral immunization has been shown to induce mucosal IgA responses.[202] The mechanism underlying this phenomenon relates to the rapid and dramatic effect the VEE particles and replicons have on the systemic innate immune response,[244] an effect that occurs in the first 24 hours after inoculation.[453] Because the stimulation of the innate immune system by VEE replicons also appears to enhance adaptive immune responses, replicons that do not express a vaccine Ag can be used as an adjuvant to enhance the protective response to subunit vaccines.[66] One of the hurdles to using alphavirus vectors is demonstrating that recombinant replication-competent viruses do not reconstitute by recombination at the extremely high inoculation concentrations requested by regulators (which exceed concentrations used in manufacturing protocols).

Herpesvirus vectors

Replication-defective HSVs have been produced in a manner analogous to that of the E1A-deficient adenoviruses, that is, they have had one or more genes deleted that are essential for replication, but replication *in vitro* is supported in a cell line that constitutively expresses the essential protein needed for replication.[109,306] In contrast to the E1-deficient adenoviruses, the replication-defective HSVs are being developed as vaccines to protect against disease caused by HSV itself, as well as a vector to deliver foreign viral proteins. Another approach to vector construction is to package replication-defective HSV amplicons in HSV particles using bacterial artificial chromosomes (BAC)-encoded helper viruses with deleted packaging sequences resulting in foreign gene expression without the influence of any residual HSV genes.[168]

Poxvirus vectors

Attenuated replication-defective poxvirus vectors have been developed to address the known complications of vaccinia virus in immunocompromised persons. The most developed vectors include modified vaccinia virus Ankara (MVA), avipox (canarypox and fowlpox), and NYVAC (deliberate deletion of 18 genes associated with virulence).[354] MVA was derived by more than 500 passages in chick embryo cells[284,296] and has been evaluated as a vaccine vector for a variety of viral Ags. MVA is a highly attenuated host-range mutant that replicates well in avian cells, has limited replication in BSC-40 and baby hamster kidney (BHK) cells, but does not propagate in most mammalian cells.[41,65,125] MVA is nonpathogenic in immunocompromised hosts and has been administered to large numbers of humans without incident.[285,439,483] Attenuation of the MVA recombinant resulted from the loss of nearly 30 kb of genome including genes for immune evasion and host range during its many passages in embryonated eggs.[12] Use of avian poxviruses, referred to as avipox viruses, as vectors represents a second type of host-range–restricted poxvirus vector. Avipox viruses are naturally occurring host-range–restricted viruses that are replication-deficient in mammalian cells and mammal hosts.[354] Each of these poxvirus vectors has the property of efficient replication *in vitro* in avian cell culture but restricted replication in primates. Avipox vectors have an added advantage because preexisting immunity to vaccinia does not impact vector delivery. Poxvirus vectors have the following advantages: (1) thermostability, (2) the history of being associated with the extraordinary success of the smallpox eradication campaign, (3) large packaging capacity for foreign genes, and (4) lack of persistence or genomic integration. The major disadvantages are (1) attenuated vectors are grown in primary cell lines (chicken embryo fibroblasts), and production is difficult to scale up; (2) the foreign gene insert is sometimes unstable; and (3) poxviruses are antigenically complex, and there may be competition between the vector and the inserted gene products for Ag processing and presentation.

After extensive phase I and phase II evaluation,[166,369] a recombinant canarypox vaccine was evaluated in a controversial phase III efficacy study, RV144.[56] This landmark study provides a benchmark as the first demonstration of efficacy for an HIV vaccine.[388] A general population cohort of 16 402 people in Thailand was enrolled in the study and randomized 1:1 vaccine to placebo recipient. Vaccinees received intramuscular injections of a recombinant canarypox vector expressing envelope, Gag, and parts of Pol and Nef given at 0, 1, 3, and 6 months and rgp120 formulated in alum given at months 3 and 6. At 18 months, 74 infections had occurred in placebo recipients and 51 infections had occurred in vaccinees, which was statistically significant. A similar rgp120 did not protect men who have sex with men[161] or intravenous drug

users,[368] populations with much higher incidence. The immune correlate of protection is not yet defined, but protection appears to be temporally associated with serum Ab titers, even though vaccinee sera do not neutralize commonly transmitted strains using traditional assays.

Limitations of Gene-Based Vectors

It is beyond the scope of this chapter to cover the growing list of potential vectors to deliver genes encoding viral Ags that may be considered for future vaccine development. For example, arenavirus[142] and lentivirus[112] vectors have not been discussed here and reverse genetic systems have been developed for other virus families[240] that will make recombinant vector development feasible. Each system has a unique set of properties that should be considered when devising a strategy for a particular pathogen. One of the primary considerations is seroprevalence for the vector in the target population. Preexisting vector-specific immunity can affect both safety and potency of the vaccine. This interaction may be a problem if there is natural immunity to the vector or if competing vaccine programs for other important pathogens converge on the same vector platforms. There are several factors involving safety, Ag selection, immunogenicity, manufacturing, and stability that are important to consider when developing a vector-based vaccine strategy that are pathogen and target population specific (e-Table 15.2).

Viral Vectors as Vaccines Against the Virus From Which the Vector Was Derived

A practical by-product of the development of gene-based replication-defective viral vaccine vectors is that the vector itself may serve as a novel vaccine platform for the virus from which the vector was derived. The most advanced examples of this are the poxvirus vectors such as MVA or NYVAC. These have been evaluated as candidate smallpox vaccines in clinical trials using replication-competent vaccinia (Dryvax) as a surrogate challenge.[152,357] This type of approach has also been proposed for herpesviruses[128] and adenoviruses. Using the viral vector creates the opportunity to express additional viral antigens[4] or immunomodulatory proteins like cytokines[75] that could provide adjuvant effects and increase immunogenicity compared to the live attenuated virus platform. Another advantage of this approach is that the vector delivers the antigenic complexity of a live attenuated virus vaccine with a more favorable risk profile because of lack of replication. In addition, the vector provides more options for achieving robust manufacturing capacity, while this is often a challenge for live attenuated viral vaccines.

Vaccine Formulation and Delivery

Adjuvants

Adaptive immune responses are controlled by the amount, duration, and formulation of the Ag in conjunction with innate immunity. The Ag sequence and structure determines specificity, but the adjuvant and context of delivery and Ag presentation determine the composition, location, kinetics, and magnitude of vaccine-induced immune responses (e-Fig. 15.8). While specificity is a property of the adaptive immune response, many adjuvant effects are mediated by the innate immune system. The most potent and broad-based adaptive immune responses are induced by replication-competent virus vaccines and gene-based viral vectors. These vectors provide efficient Ag presentation and inherently stimulate innate immunity through a variety of signaling pathways thus limiting a requirement for exogenous adjuvants. By contrast, inactivated virus vaccines, purified viral proteins, or peptide Ags by themselves have relatively weak immunogenicity in a I host. There are several reasons that replication-competent viral vaccines and gene-based vectors are inherently more immunogenic than inactivated virus or subunit vaccines. First, gene expression provides an ongoing source of the relevant protective Ag that is amplified through replication beyond the dose contained in the original inoculum. Second, Ags produced during transcription in the host cells have native quaternary and multimeric particulate structures and authentic posttranslational modifications. Third, replication-competent viruses and gene-based vectors can broadly engage multiple distinct innate immune and Ag presentation pathways. Indeed, a major advantage of live or attenuated viral vaccines is that expressed antigenic epitopes are processed efficiently through both MHC class I and II pathways. This mechanism contrasts with that of inactivated vaccines where Ag processing is mostly limited to MHC class II presentation, which elicits CD4 T-cell responses but is limited for generating CD8 immunity. Finally, PAMP recognition receptors are engaged on the cell surface, in endocytic vesicles, and in the cytoplasm with live vaccines (e-Fig. 15.9). Therefore, adjuvants are needed to compensate for the diminished immunogenicity of vaccines that are not transcriptionally active in host cells by recruiting additional signaling pathways to activate innate immunity, promoting Ag-presenting cell maturation and costimulation, and prolonging Ag persistence.

e-FIGURE 15.8 **Vaccine formulation and delivery is critical for the pattern and function of vaccine-induced immune responses.** Significant attention is given to Ag selection and design during vaccine development. While the Ag determines the specificity of the vaccine-induced immune response, many other qualities important for protective immunity depend on the formulation and delivery of the product. Formulation also is the major determinant of vaccine stability, which is a key factor in overall vaccine success.

e-FIGURE 15.9 **Adjuvant effects are mediated through pathways that recognize PAMPs.** Many of the events leading to activation of innate and adaptive immune responses begin with signaling pathways triggered by the recognition of PAMPs. Cell surface and endosomal Toll-like receptors and intracytoplasmic helicases play major roles in the recognition of virus-associated PAMPs and mediate many vaccine adjuvant effects.

Traditionally, adjuvants have been derived from natural products empirically found to have immunostimulating properties. Adjuvants can improve immunogenicity by optimizing the duration of Ag presentation through a “depot” effect and/or through enhancing certain innate stimulatory pathways. It was first discovered that bacterial toxins were more protective when mixed with other substances.[383] A prototypic adjuvant used exclusively in laboratory research is Freund adjuvant prepared from mycobacterial cell walls. A modern version of incomplete Freund adjuvant (IFA) (Montanide ISA-51) is a water-in-oil emulsion composed of mineral oil mixed with the surfactant mannose mono-oleate in a 1:1 ratio with the aqueous phase. Although vaccines formulated with mineral oil have been administered to more than 1 million people since 1945, with the emergence of aluminum-based adjuvants, they fell out of favor because of the reactogenicity profile and potential for causing sterile abscesses.[177] They are still frequently used in the context of therapeutic vaccines.

There are five currently licensed adjuvants in the United States for human use in preventive viral vaccines, (1) alum, (2) AS04—a combination of alum + monophosphoryl lipid A (MPL), (3) MF59, (4) AS01B, and (5) CpG 1018. In addition, the oil-in-water emulsion adjuvant AS03, made of vitamin E and squalene, is included in an H5N1 avian influenza vaccine in the U.S. Strategic National Stockpile.

Alum is derived from aluminum hydroxide or aluminum phosphate and is formulated as a gel or used to precipitate protein Ags to create aggregates. It functions as a short-term Ag depot in which Ag that is electrostatically bound to alum is slowly released at the site of inoculation.

Some of the adjuvant activity conferred by alum is through activation of the "inflammasome" innate signaling pathway.[131] Alum has been the standard adjuvant used in licensed vaccines for several decades. All the currently licensed inactivated viral vaccines and hepatitis B VLPs are formulated with alum. Gardasil, the Merck HPV VLP vaccine, is also formulated with alum.

Ab responses can be improved by combining additional immune stimulatory molecules to alum. Indeed, AS04 (Adjuvant System 04) is a combination of alum and a modified synthetic form of MPL, a key component of LPS that is the ligand for TLR4. Cervarix, the HPV VLP from GlaxoSmithKline, is formulated with AS04 and is the first adjuvant containing a TLR ligand to be approved for use in humans. The MPL-based adjuvants when combined either with alum (AS04) or with QS21 (AS02) have been shown to have a significant dose-sparing effect and may have additional advantages in terms of the speed and durability of the humoral responses. Formulations that include MPL also stimulate IFN-γ–producing CD4 T-cell responses, which may have additional benefit.[330]

After alum, the other most widely used adjuvant in humans is MF59. MF59 is a squalene oil-in-water emulsion that is licensed for human use in Europe. MF59 was designed originally to provide an Ag depot from which Ag would be slowly released as the biodegradable oil was metabolized or removed by macrophages and as a vehicle for the immunostimulant MTP-PE (muramyl tripeptide-phosphatidylethanolamine), a derivative of muramyl dipeptide, the active component of the mycobacterium cell wall fraction in Freund complete adjuvant. However, clinical studies in humans indicated that MF59 itself was as immunostimulatory as MF59/MTP-PE with less toxicity.[175] Its adjuvant effect is not TLR dependent, but there is evidence that MF59 has some innate immune stimulatory capacity. More than 45 million doses have now been given to humans formulated with seasonal influenza vaccines. MF59 is dose-sparing, and clinical studies with H5N1, H1N1, or seasonal trivalent influenza vaccines show that protective levels of immunity can be achieved with as little as 3.75 μg of vaccine Ag.[86] A similar squalene oil-in-water emulsion (AS03) has achieved similar dose-sparing effects when formulated with influenza Ags.[331] MF59-formulated influenza vaccines also result in antibodies with greater cross-reactivity and greater avidity to conformational epitopes than alum-formulated vaccines in head-to-head comparisons.[234] MF59 is now approved as a component of a standard-dose, inactivated quadrivalent influenza vaccine for people aged 65 years and older.

$AS01_B$ is an adjuvant system that is used to adjuvant the recombinant zoster vaccine Shingrix. $AS01_B$ uses 3-deoxy monophosphoryl lipid A (MPL) and QS21 with cholesterol included in liposomes. Cholesterol bind and detoxifies ("quenches") the toxicity of QS21 which, like other saponins, binds to cholesterol and damages cell lipid bilayer membranes. Incorporation into liposomes also eliminates the toxicity of MPL. QS21 was discovered in the bark of *Quillaja saponaria*, Molina (soap bark tree). It was named for the tree's scientific name and for being the 21st peak on the HPLC analysis of the bark extract. The basis for its adjuvant properties is incompletely understood.

The CpG 1018 adjuvant is cytosine phosphoguanine (CpG), a synthetic form of single-stranded DNA that mimics bacterial and viral genetic material and is included in a recombinant hepatitis B vaccine (Heplisav-B). Specifically, CpG is a PAMP that is recognized by the pattern recognition receptor Toll-like receptor 9 (TLR9), which is expressed only in B cells and plasmacytoid DCs.[399]

Delivery Vehicles

Delivery of vaccine Ag can determine the immunological compartment that is stimulated and influence the types of Ag-presenting cells that process the vaccine Ag. Many delivery vehicles are formulated with adjuvants or have innate adjuvant properties, but in this section, the term will refer to approaches for transporting Ag to a particular location. A delivery vehicle may allow the mucosal delivery of an Ag that could otherwise only be given parenterally. Alternatively, a delivery vehicle could function as a way of co-delivering a mixture of Ags and adjuvant, produce a depot effect, or otherwise control the timing of release of Ag. Vaccine delivery vehicles have been designed to carry Ag into subcellular compartments with the intent of stimulating specific immune responses by accessing distinct Ag-processing pathways.

Lipid-Based Carriers

Many virus surface proteins are spatially oriented in lipid envelopes and that is one rationale to use lipids in the formulation of virus vaccines. Another is that the Ag may be protected by the lipid and allow delivery through harsh environments like the gastric mucosa or blood or promote a depot effect when injected. Liposomal vaccine formulations may facilitate mRNA or DNA transport into target cells and have been used extensively for transfection of cells *in vitro*. Liposomes are vesicles with a roughly spherical lipid bilayer surrounding an aqueous center.[138] If virus-derived proteins are inserted into the membranes (proteoliposomes), they are often referred to as virosomes. Liposomes have been used to deliver peptides, proteins, whole virus, and mRNA or DNA with and without adjuvants and can be designed to target or costimulate selected APCs. The intent is to produce an inert particle that concentrates in the target organ of interest and then releases its payload. Typically, when used for purposes of gene delivery, cationic lipids are used in the liposome formulation to improve the interaction with the negatively charged nucleic acid. The lipid composition of the liposome can be modified to affect charge, pH sensitivity, thermal stability, and fusogenicity to control release of the products. Licensed mRNA vaccines for COVID-19 use lipid nanoparticle formulations, and these unique delivery systems likely form the basis for much of the immunogenicity of those vaccines. The Moderna mRNA vaccine uses a PEGylated lipid nanoparticle containing 1,2-distearoyl-sn-glycero-3-phosphocholine, cholesterol, and a proprietary synthetic lipid SM-102. The Pfizer mRNA vaccine similarly uses another unique set of four lipids to form lipid nanoparticles to protect and deliver mRNA. Virosomes are used in licensed products (non–United States) to deliver vaccine Ags for influenza and hepatitis A.[309] More elaborate virosomes in which the proteoliposome is built around a polystyrene bead are being developed as a candidate HIV-1 vaccine, using the scaffolding of the particle to produce more stable conformations of the envelope glycoprotein.[183] Exosomes are natural source of liposomes. Exosomes are 50- to 90-nm vesicles produced by an endosomal process in which vesicles bud into endosomes (multivesicular bodies) and are then secreted from APCs. Interestingly, these exocytic vesicles contain many of the proteins involved in Ag presentation including MHC and costimulatory molecules.[256] In addition, they have lipid content like many enveloped viruses and lipid rafts with high-cholesterol content.[170] ISCOMs (immune-stimulating complexes) are another lipid-based vehicle for delivering vaccine Ag. They are 40-nm cagelike structures formed by a complex mixture of lipids and saponins that can be formulated with viral Ags.[2] They can deliver proteins or peptides to the cytoplasmic compartment where they can be processed to activate CD8+ T cells, and this has also been demonstrated in clinical trials of HPV and HCV vaccine candidates.[413] ISCOMs also have adjuvant properties derived primarily from the saponin component, QS21, described above.

Synthetic Particles

Synthetic polymers have been used in creative ways to contain or hold vaccine Ags for presentation to the immune system. Microparticles are

made from biodegradable polymers complexed with viral Ags.[103] The primary polymer used is poly lactic co-glycolic acid (PLGA), which can encapsulate DNA by itself or with multiple proteins, adjuvants, or other immunomodulators. PLGA has an extensive history of safety in humans because it is the polymer used in absorbable sutures. Some other advantages of microparticles (1-10 μm) include the following: (1) preferential uptake through the phagocytic pathway of DCs, (2) protection of the vaccine product from the environment resulting in a slow-release depot effect,[342] and (3) the potential payload capacity is higher than for liposomes or viral vectors. PLGA microparticles coated with cationic surfactants have also been used as a vehicle for DNA delivery by absorption of plasmid DNA to its charged surface.[342] In general, these approaches have not produced the robust gene expression and immunogenicity needed for gene-based vaccination, and the formulations are being empirically modified. One approach has been to produce a hybrid polymer by combining PLGA with other substances like poly-β amino ester to improve the sensitivity to pH change and more rapidly liberate the DNA.[269] Another approach for DNA delivery has been to use alternative polymers such as poly (ortho esters) (POEs), which had been developed for drug and peptide delivery.[474]

Nanoparticles have been used to deliver mRNA vaccines, as discussed above, and have been explored as vehicles to deliver DNA vaccines. Generally, sizes of <200 nm (close to the size of many viruses) have been used, which are less likely to be forced into the phagolysosome pathway than microparticles. One approach has involved polylysine cationic polymers with imidazole side chains to facilitate escape from endosomes.[270] Another utilizes an oil-in-water microemulsion to produce wax cationic nanoparticles[107] that can be modified to encapsulate DNA.[318] Finally, nonionic block co-polymers have been used to deliver DNA vaccines with promising results in animal models but not in human trials.[428]

Synthetic self-assembling polypeptides can be used to display vaccine Ags on particles to achieve multivalency like the use of VLPs as Ag scaffolds.[367] Novavax developed a vaccine for COVID-19 based on baculovirus/insect cell–expressed SARS-CoV-2 spike protein incorporated into nanoparticles formed by mixing the recombinant protein in a nonionic surfactant (polysorbate 80 or Tween 80). That vaccine candidate obtained conditional marketing authorization in December 2021 from the European Commission, and it has been approved in eight countries as of April 2022 and by the FDA in July 2022. A protein nanoparticle decorated with 60 copies of the SARS-CoV-2 spike protein receptor–binding domain[471] was licensed in Korea in 2022, paving the way for increased use of nanoparticle vaccine approaches in humans. Another nanoparticle approach is to use self-assembling proteins from other (nonviral) biological sources such as the yeast protein Ty,[262] although evaluation of these structures in human trials did not induce significant immune responses.[480]

Cell-Based Carriers

Dendritic cells (DCs) are important for Ag presentation, for initiating the innate responses, and bridging the innate and adaptive immune systems to coordinate a comprehensive response to a virus infection. Therefore, they have been central to several vaccine development efforts. Not surprisingly, DCs themselves have been used as the vehicle for Ag delivery. Autologous DCs have been sorted, expanded *in vitro* with selected cytokines, pulsed with peptide, inactivated whole virus, or vectors and then reinfused into subjects.[272,281,344] Another cell-based vaccine approach utilizes transgenic plants. Potatoes, lettuce, tomatoes, carrots, bananas, and corn have all been engineered to produce vaccine Ags that have been evaluated in clinical trials.[444] This technology is also in early stages but has partially overcome some of the initial theoretical concerns that delivery of edible vaccines would result in oral tolerance or possibly result in food allergies to the carrier. A major advantage of transgenic plants is that production does not require an extensive physical infrastructure, and therefore, manufacturing is inexpensive and easily distributed.

Conjugates

Vaccines derived by conjugating protein to carbohydrate have been extraordinarily successful for the control of encapsulated bacteria. The protein in this setting helps to redirect the Ag presentation so that alternative Ab isotypes are produced to control the bacteria. Since the carbohydrate structures of viruses are made by the host cell, they are not antigenic targets and often protect the virus from host immune defenses, as exemplified by the “glycan shield” of the HIV-1 gp120. Conjugates for viral Ags have typically been designed to carry the viral Ag into the cytoplasmic compartment of the APC so that processing and MHC class I presentation and CD8+ T-cell activation can occur. This objective has been accomplished by using heat shock proteins (HSP70),[69] alpha-2 macroglobulin,[268] and leader sequences that promote membrane translocation to help the protein directly enter the cytoplasm.[205,394] Protein conjugate vaccines with Ab Fc receptors, CpG motifs, TLR 7/8 agonists[78,485] also have been used to improve the efficiency of uptake of viral Ags to DCs. An additional approach is to target Ag directly to DCs through incorporating the viral Ag into an Ab such as the DEC205 Ab conjugate that delivers Ag directly to DCs.[145,329] This provides an efficient *in vivo* approach for targeting DCs instead of the *ex vivo* approach mentioned above, which may be limited to therapeutic vaccines for chronic virus infection and cancer.

Mechanical Devices

Currently, licensed vaccines are given orally or by needle and syringe, aerosol, or bifurcated needle in the case of vaccinia. Future vaccines and adjuvants or immunomodulators may utilize more technically sophisticated devices like microneedles[237] or micromechanical abrasion.[299] Other delivery devices have been adapted or developed in large part to address the need for improving the efficacy of DNA vaccines in humans. Needle-free injection systems like Biojector or Powderject have been used to deliver DNA vaccines in clinical trials,[176,401,475] and in both animal models and humans, electroporation has been shown to improve Ag expression and immunogenicity of DNA vaccination.[416,465] In addition, there continue to be new designs for delivery by traditional aerosol or needle and syringe devices that have been designed to improve effectiveness or safety. There are rapid advances in microneedle (<1 μm) technology in which arrays of needles made from a wide variety of materials (ranging from stainless steel to dissolving crystalline sugars) can effectively deliver vaccines.

Combination Approaches

Co-delivery or Multivalent Vaccines

Co-delivery of vaccines has become a matter of practicality with more than 13 pathogens being targeted during the childhood immunization series alone. Therefore, combined vaccine formulations like MMR (measles, mumps, rubella), the tetravalent product that includes varicella vaccine with the MMR combination, IPV (polio serotypes 1, 2, and 3), and quadrivalent influenza (B and A H3N2 and H1N1) are commonly

used. Factors such as antigenic competition, interference, and unanticipated adverse reactions need to be considered during the development of combination products.

Co-delivery of different vaccines against the same pathogen can sometimes augment immunogenicity. Simultaneous administration of two different influenza virus vaccines to elderly subjects, namely, an intranasally administered live attenuated[444] influenza A virus candidate vaccine and a parenterally administered licensed inactivated virus vaccine, induced greater resistance to illness than did administration of the inactivated virus vaccine alone.[458] In this situation, the greater immunogenicity of the live virus vaccine in the respiratory tract coupled with the greater efficiency of the inactivated virus vaccine in stimulating systemic immunity appear to act in concert to optimize efficacy. A replication-defective HSV vaccine was found to be most immunogenic when administered by both parenteral and mucosal routes, indicating that the same vaccine preparation can also be given by a combination of routes to augment protection.[306]

Sequential Combination of Vaccines

Sequential combination of different vaccine modalities that focus on inducing immune responses to the same pathogen is referred to as "prime-boost" strategies.[384] This concept takes advantage of the memory inherent in the adaptive immune response by sequentially amplifying the response to selected Ags. This can be done with combinations of replication-competent or gene-based vectors with inactivated or subunit vaccine approaches or by heterologous vector combinations. Combining vaccine approaches has the potential for eliciting new components of the immune response and for broadening the response to new specificities and immunological compartments. Combining heterologous vectors has the added value of the boost avoiding preexisting antivector immunity, while amplifying the response to the expressed vaccine Ag.

Combination approaches can be used both for improving the safety of the booster vaccine and for improving immunogenicity beyond either approach alone. Polio vaccination is an example where using combined modalities improved both safety and immunogenicity. It is now recommended that only IPV be used for polio vaccination in the United States Prior to this, sequential immunization with inactivated poliovirus vaccine followed by live attenuated virus vaccine was used as a safer alternative to the three-dose live virus vaccine, OPV. The combination approach provided immunity against the rare cases of vaccine-associated polio caused by the live virus vaccine and primed for a booster effect from OPV, which expanded the immune response to the mucosal compartment. Thus, immunogenicity was optimized in the individual, and herd immunity was provided to the population by inducing sufficient mucosal immunity in the gastrointestinal tract to prevent the spread of imported strains of poliovirus.[301,366] Sequential immunization with a live attenuated alphavirus vaccine candidate followed with a boost with an inactivated virus vaccine induced higher levels of Ab than live virus alone.[370] In the field of HIV vaccine development, a variety of prime-boost strategies have been evaluated. In some cases, priming has been done with a DNA or gene-based vector followed by subunit protein boost.[91,99,174] This approach led to the first demonstration of efficacy against HIV acquisition using a recombinant canarypox vector expressing Env, Gag, and Pol boosted with recombinant gp120 protein.[388] Other approaches have utilized heterologous vector combinations or DNA priming followed by gene-based vector boosting.[9,198,247,423] These studies have shown DNA priming followed by rAd5 boosting can protect against SIV infection in macaques and reduce viral load in vaccinated animals that experience breakthrough infections.[265] This concept is currently being evaluated in a phase IIb efficacy trial (HVTN 505).

Universal or Broadly Protective Vaccines

Some viruses exhibit such antigenic diversity and drift that a single vaccine antigen is unlikely to induce broadly protective immune responses that protect against all circulating or future variants (eg, influenza viruses and coronaviruses). Recent efforts to develop more "universal" vaccines that induce protective responses against diverse variants, clades, subtypes, or even families of viruses have focused on two strategies. One is identifying and using immunogens comprising only the most conserved residues for the protective antigens (eg, influenza HA stem epitopes or coronavirus spike protein receptor–binding residues). A second strategy is to incorporate antigens from diverse antigenic variants into single immunogen preparations, such as mixtures of variant VLPs or even individual nanoparticles displaying diverse antigens. Two-component influenza nanoparticle immunogens that co-display the four hemagglutinins of licensed quadrivalent influenza vaccines have been shown to induce broad responses.[49] Co-display of hemagglutinin RBDs from multiple strains across time using adjacent RBDs that are heterotypic induces more broadly protective responses than mixtures of the same antigens on separate nanoparticles.[228]

Modernization of Vaccine Manufacturing

The process of manufacturing a vaccine is complex yet, the primary cornerstone in overall production is the substrate used for vaccine growth. Early generation vaccines were often grown in animal organ cells, including adenovirus vaccine in monkey kidney cells and JEV vaccine in mouse brains. Such tissue cultures have the potential to carry adventitious agents. In the late 1950s, a polio vaccine developed by Hilary Koprowski, was used in trials in Africa, after first being tested in the United States. A vaccine virus was given to about a million people in Burundi, Rwanda, and Democratic Republic of the Congo that had been produced in tissue cultures obtained from macaque monkey kidneys. Journalists later raised the concern that HIV was transmitted to the human population during the vaccination. Residual stocks of that vaccine were tested by independent laboratories and were confirmed to have been made using monkey (not chimpanzee) cells and no contaminations with SIV or HIV was found, so this theory has been refuted.[491] Nevertheless, high vigilance about adventitious agents is warranted when using cultures of cells.

During the second half of the 20th century, modernization of vaccine production included the progression to use of cell lines as substrates, including current JEV vaccine in Vero cells and hepatitis B vaccine in yeast. Influenza vaccine researchers continue to develop U.S.-marketed vaccines using chicken eggs as the substrate but have developed cell culture-based substrates that are used effectively in some European countries and the United States. The transition to cell-based substrates offers a more efficient, more reliable, and more stable manufacturing process. In 2009 (European Medicines Agency) or 2012 (U.S. FDA), regulatory officials approved the first mammalian cell–based influenza vaccines, quadrivalent vaccines produced in the Madin-Darby canine kidney cell line. Production of influenza antigens in mammalian cells has the advantage of better mimicking virus replicating in human cells and avoiding egg-adaptation mutations that occur in egg propagation and reduce the immunogenicity for certain protective epitopes.

Impact of Advances in High-Throughput Sequencing Technology on Quality Control

The human genome project simulated major investments in sequencing technology, resulting in breakthroughs that have increased throughput

by orders of magnitude. As a result, it is now feasible to sequence all nucleic acids in samples of interest, such as vaccine preparations. Not surprisingly, such experiments have identified previously unidentified agents or nucleic acids in licensed vaccines, such as porcine circovirus-1 (PCV-1) in the commercial rotavirus vaccine Rotarix.[468] The FDA suspended use of the vaccine in 2010; however, experts agreed this highly prevalent nonpathogenic pig virus does not infect humans, and the use of vaccine was reinstituted. Porcine circovirus–free formulations of human rotavirus vaccine have now been prepared and large multicountry clinical studies have shown that these formulations had similar reactogenicity and safety profiles.[260,410]

Some vaccines are produced in avian or primate cell lines, and thus, it was not surprising that RNAs from avian leukosis virus, which is noninfectious for humans, or genetically defective DNAs from simian retrovirus (SRV), were present in products of such cell lines. Another interesting finding of such sequencing efforts is that the vaccine virus populations contain minority variants with polymorphisms or mutations (compared to the known sequence of the vaccine strains). Such variants have been observed for OPV, mumps virus, and varicella-zoster viruses, but they are not known to affect attenuation. First-generation smallpox vaccines were derived from uncloned viruses propagated in calf-lymph, including Dryvax, Aventis Pasteur Smallpox Vaccine (APVS), Lister, and Lancy-Vaxina. The smallpox vaccine Dryvax, first licensed by the FDA in 1931, contained clones with heterogeneous sequences, sometimes resulted in shedding of mouse-neurovirulent clones, and is no longer manufactured. ACAM2000 is a second-generation smallpox vaccine based on a clone isolated from Dryvax, which is purified and produced using modern cell culture. ACAM2000 differs from the Dryvax consensus sequence by single nucleotide polymorphisms and insertions-deletions of varied size.[346]

The emergency status declared during the COVID-19 pandemic starting in 2020 spurred the most rapid pace of vaccine development for an agent in history. The emerging technology of mRNA and rAd-vectored vaccines facilitated design of some vaccine candidates within hours of the public release of an SARS-CoV-2 spike protein gene sequence in January 2020. The first clinical trial of an mRNA vaccine for COVID-19 was initiated by March 16, 2020, only weeks later. This rapidity of development required new technologies, rapid manufacturing processes, expedited regulatory review, and many other innovations, and ultimately led to rapid approval for several COVID-19 vaccines. Whether lessons learned in these successful ultrarapid campaigns can be translated to time saved in future nonpandemic efforts remains to be seen.

Maintaining Vaccine Availability

Vaccine efficacy, need, utilization, and safety (or perceived safety) all have a significant impact on the success of licensure and sustained availability of a vaccine. Production of the adenovirus vaccine for serotypes 4 and 7 that is selectively given to new military recruits entering basic training was stopped by the sole manufacturer in 1995 for economic reasons, and an outbreak of acute respiratory disease hospitalizations occurred in basic training soldiers almost immediately when vaccination was ceased.[29] Morbidity from adenovirus infections increased among military recruits, and epidemics occurred in training centers.[180] By 2011, the government reinstituted immunization with newly licensed oral vaccine from a new manufacturer. In addition to economic concerns, safety concerns have also prompted an abrupt end to the use of vaccines. Specifically, Rotashield, a live tetravalent rhesus rotavirus reassortant vaccine against the four major prevalent serotypes (based on VP7, a major outer capsid glycoprotein) was effective but was later withdrawn from market. A postmarketing analysis revealed that the vaccine was associated with an excess rate of intussusception of up to 1 per 2500 vaccinees that occurred in the immediate postvaccination period, predominantly following the first dose of vaccine.[320] Following withdrawal of Rotashield, subsequent studies led to a significant downward revision of these estimates and have suggested that the early period of increase in intussusception seen after the first dose was followed by a period of compensatory decrease such that there was no net overall increase in the number of cases in vaccinees <1 year of age.[320] Furthermore, the excess cases of intussusception occurred mostly when the vaccine was used in children 3 months of age or older to “catch up” rather than in vaccinees who received the ideally recommended schedule of 2, 4, and 6 months of age.[229,432] This case highlights the importance of the analysis of postmarketing data, which needs to be especially thorough and comprehensive because it can dramatically impact the availability of a vaccine that may otherwise have a favorable risk/benefit ratio. Despite the initial conclusions and withdrawal of Rotashield from the market, two newer versions of a rotavirus vaccine were developed. Pentavalent RotaTeq has been licensed in the United States for infants <3 months of age. It consists of a mixture of live attenuated, bovine/human reassortant viruses that express a single VP4 (P1A) as well as VP7 (G) Ag from G1-G4 strains of rotavirus [8]. The second vaccine, Rotarix, is a monovalent VP7 G1:P1A [8] attenuated human rotavirus strain that was first licensed in Mexico and the Dominican Republic,[165] and later in 2008 in the United States. These two vaccines protect against severe gastroenteritis due to rotavirus. Large-scale postlicensure evaluations were conducted to more clearly elucidate the overall risks associated with rotavirus vaccines in the general population. In these studies, cases of intussusception were observed in temporal association within 21 (RotaTeq) or 31 (Rotarix) days following the first dose, with clustering of cases in the first 7 days. These findings translate to potentially 0-4 additional cases of intussusception hospitalizations per 100 000 vaccinated infants within 31 days after the first dose in the United States.

Influences on Vaccine Uptake

Government policies and the recommendations of professional societies greatly affect the use of vaccines. The U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) are some of the principal agencies that license (allow or authorize) vaccines for human use but then additional bodies of experts provide recommendations for use. In the United States, for example, the Advisory Committee on Immunization Practices (ACIP) of the U.S. Centers for Disease Control and Prevention (CDC), the American Academy of Pediatrics (AAP), the American Academy of Family Physicians (AAFP), and the American College of Obstetricians and Gynecologists (ACOG) publish influential recommendations. Third-party payers (such as private insurance companies, Medicare, and Medicaid) also influence vaccine use through reimbursement policies. Even when vaccines are licensed and recommended, uptake is rarely universal without legal requirements (such as school-entry requirements). The public exhibits varying levels of interest in vaccination and trust in public health official recommendations. Many people delay their acceptance or refuse vaccination despite availability of vaccination services for themselves or their family members, a state that has been termed “vaccine hesitancy.” Vaccine hesitancy stems from a variety of concerns and operates differently in varying contexts. Some people lack sufficient knowledge about the vaccine or disease in question, some have overconfidence in their natural ability to resist severe disease, and some lack confidence in the benefits of vaccination. Practical fears arise, such as anxiety about needles or the complexity of medical settings or concerns about potential side effects, real or imagined. On top of these medical issues, misinformation campaigns propagated by individuals or groups who oppose vaccination have left many in the public with an implicit association of vaccines with potential for harm. Also, when individuals do not perceive virus infections to be a threat to them, they fail to get vaccinated due to complacency. Vaccine hesitancy may also stem from broad distrust of the general medical system, a state that may be exacerbated by legitimate concerns of historical or current racial and economic disparities in health care access or practice. Less common is total vaccine refusal (so-called anti-vax sentiments) that stem from political, social, emotional origins that are difficult to address and exacerbated by social media disinformation and even malicious foreign government disinformation campaigns intended to introduce conflict and instability in societies.

Summary

Vaccine development has occurred primarily through observation and empiricism. In some cases, the emergence of new technologies allowed advances in vaccine development, and occasionally, basic research discoveries have led to successful new vaccine approaches. The earliest observations that smallpox did not occur twice in the same individual led to the development of insufflation and variolization. Observing that a relatively innocuous cowpox infection seemed to protect against smallpox led Jenner to vaccinate. When Pasteur observed that an aged bacterial culture did not cause disease in chickens, and subsequently found that those chickens were protected from challenge with fresh cultures, an era of intentional modification and attenuation of microbes began that led to the introduction of many new vaccines. New technologies, such as cell culture, led to a series of new vaccines because viruses like measles, mumps, rubella, and polio could be cultivated and produced in large quantities. The advent of molecular biology has led to the production of subunit proteins for successful hepatitis B and HPV vaccines and the technical ability to produce reassortant and recombinant viruses. Modern immunology, driven largely by the response to the HIV/AIDS crisis, has introduced new methods for measuring T-cell function that will help define the role of T cells in vaccine-induced immunity and guide the use of molecular adjuvants. Structural biology is providing an increasingly rapid way to view the atomic structures of viruses and their antigenic determinants bound to antibodies and suggesting new approaches for vaccine design. The expanded capacity for nucleic acid sequencing has facilitated the discovery and surveillance of viral threats and has provided new insights to the biology of viruses and immune effectors. Technological advances in engineering and manufacturing have made vaccines safer and have created many new approaches for delivery that will have additional benefits on safety, stability, and ease of administration.

Vaccine development typically has required decades to accomplish and has never been a process that could occur in response to a crisis. However, a better awareness of the inevitability of new emerging infectious diseases and heightened concerns about bioterrorism has challenged traditional processes and compelled the consideration of new approaches. For example, the FDA has created the “Two Animal Rule,” which is a provision for vaccine licensure based on efficacy data in animal models combined with safety data in humans for diseases that are not conducive to efficacy trials.[442] A test case for the ability to develop vaccines rapidly for emerging infectious diseases occurred during the outbreak of the SARS coronavirus (CoV). This disease was recognized in the winter of 2003, the virus was identified in March 2003, a full-length sequence was obtained in April 2003, and vaccine development of whole inactivated, vector-based, DNA, and subunit vaccines began immediately. Phase I clinical trials of the whole-inactivated virus vaccine began in the summer of 2004 and DNA trials started in December 2004. Fortunately, by that time, the epidemic had been contained through classical public health approaches of surveillance and quarantine. In 2009, when the pandemic H1N1/A/California/2009 virus appeared, both commercial licensed approaches and some experimental vaccines were in clinical use or testing within 4 months. During the onset of the COVID-19 pandemic, rapid development of highly effective mRNA vaccines was conducted with lightning speed, with the first vaccine approved by the U.S. FDA 11 months after the first case in the United States. Just as toxoids and polysaccharide conjugates have been successful platform technologies for toxin-producing and encapsulated bacteria, respectively, patterns are emerging for vaccine approaches that have a high likelihood of success against some types of viruses. For example, using reassortment to produce live attenuated vaccines for viruses with segmented genomes, or producing VLPs with the capsid proteins of nonenveloped and some enveloped viruses appear to be viable platforms for those types of viruses. While this chapter has focused on antiviral vaccines for human pathogens, much can be learned from the development of veterinary vaccines (e-Table 15.3). This list of licensed veterinary antiviral vaccines is shown to demonstrate the enormous variety of viruses to which vaccines have been developed, the types of approaches that have had some measure of success against various virus families, and to illustrate that the introduction of gene-based vaccine approaches into veterinary practice is anticipating the evolution of new human vaccines (e-Table 15.3).

The goal for future vaccine development is to identify preventive strategies for selected pathogens that cause a large disease burden (eg, HIV-1, RSV, dengue, and HSV), that have the potential for sporadic epidemics or intentional release and high disease severity (eg, orthopoxviruses, filoviruses, flaviviruses), or emerging pathogens with the potential for causing widespread epidemics (eg, chikungunya or H5N1 influenza), and at the same time, develop platform technologies that can be deployed more rapidly when new virus diseases emerge (eg, SARS-CoV and West Nile virus). A major goal in the field is development of more broadly protective or universal vaccines for antigenically variable viruses including influenza and coronaviruses. Large-scale “prototype pathogens” research programs are being launched with the goal to develop prototype vaccines for pathogens that elucidate immunity principles for entire classes of viruses. In the case of an epidemic, such principles learned with one representative pathogen might be used to rapidly develop new vaccine for emerging or new viruses in that same class or family. The 21st century promises higher capacity for protein production, structure-based Ag design, molecular adjuvants, and delivery devices to direct immune response patterns, a better understanding of the genetics of human immune response polymorphisms and repertoire, and more complete knowledge of immune effector mechanisms. In addition, the safety and efficacy of several classes of gene-based delivery vectors will be determined. These advances will hopefully provide the knowledge and infrastructure needed to develop vaccines for new emerging viruses and for difficult viruses that have eluded vaccine solutions and continue to impact human health. The optimal implementation of new vaccine strategies and informing individuals with vaccine hesitancy will require full engagement of scientists and public health authorities with the public. Clear and consistent communication about the risks and benefits of vaccines is needed. Clarifying the central goal of most vaccine programs (protection from severe disease but not always mild disease or infection) will be important. Increasing equitable access to vaccines for all people at risk is a major goal of current public health efforts.

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REFERENCES

1. Ada GL, Leung KN, Ertl H. An analysis of effector T cell generation and function in mice exposed to influenza A or Sendai viruses. Immunol Rev. 1981;58:5-24.

2. Afzal MA, Pickford AR, Forsey T, Heath AB, Minor PD. The Jeryl Lynn vaccine strain of mumps virus is a mixture of two distinct isolates. J Gen Virol. 1993;74(Pt 5):917-920.

3. Akahata W, Yang ZY, Andersen H, et al. A virus-like particle vaccine for epidemic Chikungunya virus protects nonhuman primates against infection. Nat Med. 2010;16(3):334-338.

4. Akhrameyeva NV, Zhang P, Sugiyama N, Behar SM, Yao F. Development of a glycoprotein D-expressing dominant-negative and replication-defective herpes simplex virus 2 (HSV-2) recombinant viral vaccine against HSV-2 infection in mice. J Virol. 2011;85(10):5036-5047.

5. Albrecht P, Ennis FA, Saltzman EJ, Krugman S. Persistence of maternal antibody in infants beyond 12 months: mechanism of measles vaccine failure. J Pediatr. 1977;91(5):715-718.

6. Aldovini A, Young RA. Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. Nature. 1991;351(6326):479-482.

7. Allen JD, Jang H, DiNapoli J, Kleanthous H, Ross TM. Elicitation of protective antibodies against 20 years of future H3N2 cocirculating influenza virus variants in ferrets preimmune to historical H3N2 influenza viruses. J Virol. 2019;93(3):e00946-18.

8. Alwan WH, Record FM, Openshaw PJ. CD4+ T cells clear virus but augment disease in mice infected with respiratory syncytial virus. Comparison with the effects of CD8+ T cells. Clin Exp Immunol. 1992;88(3):527-536.

9. Amara RR, Villinger F, Altman JD, et al. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science. 2001;292(5514):69-74.

10. Anderson JJ, Norden J, Saunders D, Toms GL, Scott R. Analysis of the local and systemic immune responses induced in BALB/c mice by experimental respiratory syncytial virus infection. J Gen Virol. 1990;71:1561-1570.

11. Andino R, Silvera D, Suggett SD, et al. Engineering poliovirus as a vaccine vector for the expression of diverse antigens. Science. 1994;265(5177):1448-1451.

12. Antoine G, Scheiflinger F, Dorner F, Falkner FG. The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. Virology. 1998;244(2):365-396.

13. Armstrong SJ, Outlaw MC, Dimmock NJ. Morphological studies of the neutralization of influenza virus by IgM. J Gen Virol. 1990;71(Pt 10):2313-2319.

14. Arness MK, Eckart RE, Love SS, et al. Myopericarditis following smallpox vaccination. Am J Epidemiol. 2004;160(7):642-651.

15. Arvin AM, Fink K, Schmid MA, et al. A perspective on potential antibody-dependent enhancement of SARS-CoV-2. Nature. 2020;584(7821):353-363.

16. Assessment of adenoviral vector safety and toxicity: report of the National Institutes of Health Recombinant DNA Advisory Committee. Hum Gene Ther. 2002;13(1):3-13.

17. August A, Attarwala HZ, Himansu S, et al. A phase 1 trial of lipid-encapsulated mRNA encoding a monoclonal antibody with neutralizing activity against Chikungunya virus. Nat Med. 2021;27(12):2224-2233.

18. Baden LR, El Sahly HM, Essink B, et al. Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. N Engl J Med. 2021;384(5):403-416.

19. Baldridge JR, McGraw TS, Paoletti A, Buchmeier MJ. Antibody prevents the establishment of persistent arenavirus infection in synergy with endogenous T cells. J Virol. 1997;71(1):755-758.

20. Balke I, Zeltins A. Recent advances in the use of plant virus-like particles as vaccines. Viruses. 2020;12(3):270.

21. Bangaru S, Lang S, Schotsaert M, et al. A site of vulnerability on the influenza virus hemagglutinin head domain trimer interface. Cell. 2019;177(5):1136-1152 e18.

22. Bangaru S, Zhang H, Gilchuk IM, et al. A multifunctional human monoclonal neutralizing antibody that targets a unique conserved epitope on influenza HA. Nat Commun. 2018;9(1):2669.

23. Banks WA. From blood-brain barrier to blood-brain interface: new opportunities for CNS drug delivery. Nat Rev Drug Discov. 2016;15(4):275-292.

24. Bansal GP, Hatfield JA, Dunn FE, et al. Candidate recombinant vaccine for human B19 parvovirus. J Infect Dis. 1993;167(5):1034-1044.

25. Barbas III CF, Burton DR, Scott JK, Silverman GJ. Phage Display: A Laboratory Manual. Cold Spring Harbor Laboratory Press; 2001.

26. Barouch DH, Kunstman J, Kuroda MJ, et al. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature. 2002;415(6869):335-339.

27. Barouch DH, Nabel GJ. Adenovirus vector-based vaccines for human immunodeficiency virus type 1. Hum Gene Ther. 2005;16(2):149-156.

28. Barouch DH, O'Brien KL, Simmons NL, et al. Mosaic HIV-1 vaccines expand the breadth and depth of cellular immune responses in rhesus monkeys. Nat Med. 2010;16(3):319-323.

29. Barraza EM, Ludwig SL, Gaydos JC, Brundage JF. Reemergence of adenovirus type 4 acute respiratory disease in military trainees: report of an outbreak during a lapse in vaccination. J Infect Dis. 1999;179(6):1531-1533.

30. Baxter R, Patriarca PA, Ensor K, Izikson R, Goldenthal KL, Cox MM. Evaluation of the safety, reactogenicity and immunogenicity of FluBlok(R) trivalent recombinant baculovirus-expressed hemagglutinin influenza vaccine administered intramuscularly to healthy adults 50-64 years of age. Vaccine. 2011;29(12):2272-2278.

31. Beasley RP, Hwang LY, Stevens CE, et al. Efficacy of hepatitis B immune globulin for prevention of perinatal transmission of the hepatitis B virus carrier state: final report of a randomized double-blind, placebo-controlled trial. Hepatology. 1983;3(2):135-141.

32. Beeler JA, van Wyke Coelingh K. Neutralization epitopes of the F glycoprotein of respiratory syncytial virus: effect of mutation upon fusion function. J Virol. 1989;63(7):2941-2950.

33. Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. Nat Immunol. 2005;6(4):353-360.

34. Ben-Yedidia T, Marcus H, Reisner Y, Arnon R. Intranasal administration of peptide vaccine protects human/mouse radiation chimera from influenza infection. Int Immunol. 1999;11(7):1043-1051.

35. Beran J. Safety and immunogenicity of a new hepatitis B vaccine for the protection of patients with renal insufficiency including pre-haemodialysis and haemodialysis patients. Expert Opin Biol Ther. 2008;8(2):235-247.

36. Beran J, Hobzova L, Wertzova V, et al. Safety and immunogenicity of an investigational adjuvanted hepatitis B vaccine (HB-AS02V) in healthy adults. Hum Vaccin. 2010;6(7):578-584.

37. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science. 2002;298(5601):2199-2202.

38. Bernstein DI, Stanberry LR. Herpes simplex virus vaccines. Vaccine. 1999;17(13-14):1681-1689.

39. Biering SB, Akey DL, Wong MP, et al. Structural basis for antibody inhibition of flavivirus NS1-triggered endothelial dysfunction. Science. 2021;371(6525):194-200.

40. Billam P, Bonaparte KL, Liu J, et al. T Cell receptor clonotype influences epitope hierarchy in the CD8+ T cell response to respiratory syncytial virus infection. J Biol Chem. 2011;286(6):4829-4841.

41. Blanchard TJ, Alcami A, Andrea P, Smith GL. Modified vaccinia virus Ankara undergoes limited replication in human cells and lacks several immunomodulatory proteins: implications for use as a human vaccine. J Gen Virol. 1998;79:1159-1167.

42. Blattman JN, Antia R, Sourdive DJ, et al. Estimating the precursor frequency of naive antigen-specific CD8 T cells. J Exp Med. 2002;195(5):657-664.

43. Bommakanti G, Citron MP, Hepler RW, et al. Design of an HA2-based *Escherichia coli* expressed influenza immunogen that protects mice from pathogenic challenge. Proc Natl Acad Sci U S A. 2010;107(31):13701-13706.

44. Bomsel M, Tudor D, Drillet AS, et al. Immunization with HIV-1 gp41 subunit virosomes induces mucosal antibodies protecting nonhuman primates against vaginal SHIV challenges. Immunity. 2011;34(2):269-280.

45. Borisova GP, Berzins I, Pushko PM, et al. Recombinant core particles of hepatitis B virus exposing foreign antigenic determinants on their surface. FEBS Lett. 1989;259(1):121-124.

46. Bostic JR, Brown KE, Young NS, Koenig S. Quantitative analysis of neutralizing immune responses to human parvovirus B19 using a novel reverse transcriptase-polymerase chain reaction-based assay. J Infect Dis. 1999;179(3):619-626.

47. Bouchard MJ, Lam DH, Racaniello VR. Determinants of attenuation and temperature sensitivity in the type 1 poliovirus Sabin vaccine. J Virol. 1995;69(8):4972-4978.

48. Bournazos S, Ravetch JV. Fcgamma receptor function and the design of vaccination strategies. Immunity. 2017;47(2):224-233.

49. Boyoglu-Barnum S, Ellis D, Gillespie RA, et al. Quadrivalent influenza nanoparticle vaccines induce broad protection. Nature. 2021;592(7855):623-628.

50. Brecht H, Hammerling U, Rott R. Undisturbed release of influenza virus in the presence of univalent antineuraminidase antibodies. Virology. 1971;46(2):337-343.

51. Brockman MA, Knipe DM. Herpes simplex virus vectors elicit durable immune responses in the presence of preexisting host immunity. J Virol. 2002;76(8):3678-3687.

52. Brough DE, Lizonova A, Hsu C, Kulesa VA, Kovesdi I. A gene transfer vector-cell line system for complete functional complementation of adenovirus early regions E1 and E4. J Virol. 1996;70(9):6497-6501.

53. Buchbinder SP, Mehrotra DV, Duerr A, et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. Lancet. 2008;372(9653):1881-1893.

54. Burns JW, Siadat-Pajouh M, Krishnaney AA, Greenberg HB. Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity. Science. 1996;272(5258):104-107.

55. Burton DR. What are the most powerful immunogen design vaccine strategies? Reverse vaccinology 2.0 shows great promise. Cold Spring Harb Perspect Biol. 2017;9(11):a030262.

56. Burton DR, Desrosiers RC, Doms RW, et al. Public health. A sound rationale needed for phase III HIV-1 vaccine trials. Science. 2004;303(5656):316.

57. Burton EA, Fink DJ, Glorioso JC. Gene delivery using herpes simplex virus vectors. DNA Cell Biol. 2002;21(12):915-936.

58. Buser F. Side reaction to measles vaccination suggesting the Arthus phenomenon. N Engl J Med. 1967;277(5):250-251.

59. Butman BT, Lizonova A, Brough DE, et al. Comprehensive characterization of the 293-ORF6 cell line. Dev Biol (Basel). 2006;123:225-233; discussion 265-266.

60. Calain P, Roux L. Generation of measles virus defective interfering particles and their presence in a preparation of attenuated live-virus vaccine. J Virol. 1988;62(8):2859-2866.

61. Calarese DA, Lee HK, Huang CY, et al. Dissection of the carbohydrate specificity of the broadly neutralizing anti-HIV-1 antibody 2G12. Proc Natl Acad Sci U S A. 2005;102(38):13372-13377.

62. Cannon MJ, Openshaw PJ, Askonas BA. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J Exp Med. 1988;168:1163-1168.

63. Cannon MJ, Stott EJ, Taylor G, Askonas BA. Clearance of persistent respiratory syncytial virus infections in immunodeficient mice following transfer of primed T cells. Immunology. 1987;62(1):133-138.

64. Cao L, Coventry B, Goreshnik I, et al. Design of protein binding proteins from target structure alone. Nature. 2022;605(7910):551-560.

65. Carroll MW, Moss B. Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: propagation and generation of recombinant viruses in a nonhuman mammalian cell line. Virology. 1997;238:198-211.

66. Carroll TD, Matzinger SR, Barro M, et al. Alphavirus replicon-based adjuvants enhance the immunogenicity and effectiveness of Fluzone (R) in rhesus macaques. Vaccine. 2011;29(5):931-940.

67. Casazza JP, Bowman K, Adzuku S, et al. Therapeutic vaccination expands and improves the function of the HIV-specific memory T cell repertoire. J Infect Dis. 2013;207(12):1829-1840.

68. Castanon S, Marin MS, Martin-Alonso JM, et al. Immunization with potato plants expressing VP60 protein protects against rabbit hemorrhagic disease virus. J Virol. 1999;73(5):4452-4455.

69. Castellino F, Boucher PE, Eichelberg K, et al. Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. J Exp Med. 2000;191(11):1957-1964.

70. Catanzaro AT, Roederer M, Koup RA, et al. Phase I clinical evaluation of a six-plasmid multiclade HIV-1 DNA candidate vaccine. Vaccine. 2007;25(20):4085-4092.

71. Cecchini S, Negrete A, Virag T, Graham BS, Cohen JI, Kotin RM. Evidence of prior exposure to human bocavirus as determined by a retrospective serological study of 404 serum samples from adults in the United States. Clin Vaccine Immunol. 2009;16(5):597-604.

72. Chang MH, You SL, Chen CJ, et al. Decreased incidence of hepatocellular carcinoma in hepatitis B vaccinees: a 20-year follow-up study. J Natl Cancer Inst. 2009;101(19):1348-1355.

73. Chanock RM, Ludwig W, Heubner RJ, Cate TR, Chu LW. Immunization by selective infection with type 4 adenovirus grown in human diploid tissue cultures. I. Safety and lack of oncogenicity and tests for potency in volunteers. JAMA. 1966;195(6):445-452.

74. Chantler JK, Ford DK, Tingle AJ. Persistent rubella infection and rubella-associated arthritis. Lancet. 1982;1(8285):1323-1325.

75. Chavan R, Marfatia KA, An IC, Garber DA, Feinberg MB. Expression of CCL20 and granulocyte-macrophage colony-stimulating factor, but not Flt3-L, from modified vaccinia virus ankara enhances antiviral cellular and humoral immune responses. J Virol. 2006;80(15):7676-7687.

76. Chen BJ, Leser GP, Morita E, Lamb RA. Influenza virus hemagglutinin and neuraminidase, but not the matrix protein, are required for assembly and budding of plasmid-derived virus-like particles. J Virol. 2007;81(13):7111-7123.

77. Chen X, Rock MT, Hammonds J, et al. Pseudovirion particle production by live poxvirus human immunodeficiency virus vaccine vector enhances humoral and cellular immune responses. J Virol. 2005;79(9):5537-5547.

78. Cho HJ, Takabayashi K, Cheng PM, et al. Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. Nat Biotechnol. 2000;18(5):509-514.

79. Choe H, Li W, Wright PL, et al. Tyrosine sulfation of human antibodies contributes to recognition of the CCR5 binding region of HIV-1 gp120. Cell. 2003;114(2):161-170.

80. Chothia C, Lesk AM. Canonical structures for the hypervariable regions of immunoglobulins. J Mol Biol. 1987;196(4):901-917.

81. Chumakov KM, Norwood LP, Parker ML, Dragunsky EM, Ran YX, Levenbook IS. RNA sequence variants in live poliovirus vaccine and their relation to neurovirulence. J Virol. 1992;66(2):966-970.

82. Chun TW, Justement JS, Moir S, et al. Suppression of HIV replication in the resting CD4+ T cell reservoir by autologous CD8+ T cells: implications for the development of therapeutic strategies. Proc Natl Acad Sci U S A. 2001;98(1):253-258.

83. Chung AW, Alter G. Systems serology: profiling vaccine induced humoral immunity against HIV. Retrovirology. 2017;14(1):57.

84. Chung AW, Ghebremichael M, Robinson H, et al. Polyfunctional Fc-effector profiles mediated by IgG subclass selection distinguish RV144 and VAX003 vaccines. Sci Transl Med. 2014;6(228):228ra38.

85. Chung CY, Chen CY, Lin SY, et al. Enterovirus 71 virus-like particle vaccine: improved production conditions for enhanced yield. Vaccine. 2010;28(43):6951-6957.

86. Clark TW, Pareek M, Hoschler K, et al. Trial of 2009 influenza A (H1N1) monovalent MF59-adjuvanted vaccine. N Engl J Med. 2009;361(25):2424-2435.

87. Clarke BE, Sangar DV. Processing and assembly of foot-and-mouth disease virus proteins using subgenomic RNA. J Gen Virol. 1988;69(Pt

9):2313-2325.

88. Clemens DL, Wolfinbarger JB, Mori S, Berry BD, Hayes SF, Bloom ME. Expression of Aleutian mink disease parvovirus capsid proteins by a recombinant vaccinia virus: self-assembly of capsid proteins into particles. J Virol. 1992;66(5):3077-3085.

89. Clements ML, Betts RF, Tierney EL, Murphy BR. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. J Clin Microbiol. 1986;24(1):157-160.

90. Clements ML, Makhene MK, Karron RA, et al. Effective immunization with live attenuated influenza A virus can be achieved in early infancy. Pediatric Care Center. J Infect Dis. 1996;173(1):44-51.

91. Clements-Mann ML, Weinhold K, Matthews TJ, et al. Immune responses to human immunodeficiency virus (HIV) type 1 induced by canarypox expressing HIV-1MN gp120, HIV-1SF2 recombinant gp120, or both vaccines in seronegative adults. NIAID AIDS Vaccine Evaluation Group. J Infect Dis. 1998;177(5):1230-1246.

91a. Cochi SL, Prebud SR, Orenstein WA. Perspectives on the relative resurgence of mumps in the United States. *Am J Dis Child*. 1988;142:499-507.

92. Coelingh KJ, Winter CC, Murphy BR, et al. Conserved epitopes on the hemagglutinin-neuraminidase proteins of human and bovine parainfluenza type 3 viruses: nucleotide sequence analysis of variants selected with monoclonal antibodies. J Virol. 1986;60(1):90-96.

93. Coelingh KV, Winter CC. Naturally occurring human parainfluenza type 3 viruses exhibit divergence in amino acid sequence of their fusion protein neutralization epitopes and cleavage sites. J Virol. 1990;64(3):1329-1334.

94. Coleman JR, Papamichail D, Skiena S, Futcher B, Wimmer E, Mueller S. Virus attenuation by genome-scale changes in codon pair bias. Science. 2008;320(5884):1784-1787.

95. Collins JJ, Sackie DM, Johnson GR. Immunotherapy of murine leukemia. IX. The requirement for the Fc portion of antibody for successful passive serum therapy of Friend leukemia virus-induced disease. Virology. 1983;126(1):259-266.

96. Connors M, Collins PL, Firestone CY, et al. Cotton rats previously immunized with a chimeric RSV FG glycoprotein develop enhanced pulmonary pathology when infected with RSV, a phenomenon not encountered following immunization with vaccinia—RSV recombinants or RSV. Vaccine. 1992;10(7):475-484.

97. Conrad ME, Lemon SM. Prevention of endemic icteric viral hepatitis by administration of immune serum gamma globulin. J Infect Dis. 1987;156(1):56-63.

98. Centers for Disease Control. https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-classifications.html#concern

99. Cooney EL, McElrath MJ, Corey L, et al. Enhanced immunity to human immunodeficiency virus (HIV) envelope elicited by a combined vaccine regimen consisting of priming with a vaccinia recombinant expressing HIV envelope and boosting with gp160 protein. Proc Natl Acad Sci U S A. 1993;90(5):1882-1886.

100. Cooper CL, Angel JB, Seguin I, Davis HL, Cameron DW. CPG 7909 adjuvant plus hepatitis B virus vaccination in HIV-infected adults achieves long-term seroprotection for up to 5 years. Clin Infect Dis. 2008;46(8):1310-1314.

101. Corbeil S, Seguin C, Trudel M. Involvement of the complement system in the protection of mice from challenge with respiratory syncytial virus long strain following passive immunization with monoclonal antibody 18A2B2. Vaccine. 1996;14(6):521-525.

102. Correia BE, Ban YE, Holmes MA, et al. Computational design of epitope-scaffolds allows induction of antibodies specific for a poorly immunogenic HIV vaccine epitope. Structure. 2010;18(9):1116-1126.

103. Cox JC, Coulter AR. Adjuvants—a classification and review of their modes of action. Vaccine. 1997;15(3):248-256.

104. Crotty S. Follicular helper CD4 T cells (TFH). Annu Rev Immunol. 2011;29:621-663.

105. Crowe JE Jr, Bui PT, Siber GR, Elkins WR, Chanock RM, Murphy BR. Cold-passaged, temperature-sensitive mutants of human respiratory syncytial virus (RSV) are highly attenuated, immunogenic, and protective in seronegative chimpanzees, even when RSV antibodies are infused shortly before immunization. Vaccine. 1995;13(9):847-855.

106. Crowe JE Jr, Firestone CY, Murphy BR. Passively acquired antibodies suppress humoral but not cell-mediated immunity in mice immunized with live attenuated respiratory syncytial virus vaccines. J Immunol. 2001;167(7):3910-3918.

107. Cui Z, Mumper RJ. Genetic immunization using nanoparticles engineered from microemulsion precursors. Pharm Res. 2002;19(7):939-946.

108. Cunningham AL, Lal H, Kovac M, et al. Efficacy of the herpes zoster subunit vaccine in adults 70 years of age or older. N Engl J Med. 2016;375(11):1019-1032.

109. Da Costa XJ, Jones CA, Knipe DM. Immunization against genital herpes with a vaccine virus that has defects in productive and latent infection. Proc Natl Acad Sci U S A. 1999;96(12):6994-6998.

110. D'Aoust MA, Couture MM, Charland N, et al. The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza. Plant Biotechnol J. 2010;8(5):607-619.

111. Dagan R, Amir J, Mijalovsky A, et al. Immunization against hepatitis A in the first year of life: priming despite the presence of maternal antibody. Pediatr Infect Dis J. 2000;19(11):1045-1052.

112. Dai B, Yang L, Yang H, Hu B, Baltimore D, Wang P. HIV-1 Gag-specific immunity induced by a lentivector-based vaccine directed to dendritic cells. Proc Natl Acad Sci U S A. 2009;106(48):20382-20387.

113. Dai K, Liu Y, Liu M, et al. Pathogenicity and immunogenicity of recombinant Tiantan Vaccinia Virus with deleted C12L and A53R genes. Vaccine. 2008;26(39):5062-5071.

114. Dall'Acqua WF, Kiener PA, Wu H. Properties of human IgG1s engineered for enhanced binding to the neonatal Fc receptor (FcRn). J Biol Chem. 2006;281(33):23514-23524.

115. Day PM, Kines RC, Thompson CD, et al. In vivo mechanisms of vaccine-induced protection against HPV infection. Cell Host Microbe. 2010;8(3):260-270.

116. De Rosa SC, Lu FX, Yu J, et al. Vaccination in humans generates broad T cell cytokine responses. J Immunol. 2004;173(9):5372-5380.

117. De Wit D, Olislagers V, Goriely S, et al. Blood plasmacytoid dendritic cell responses to CpG oligodeoxynucleotides are impaired in human newborns. Blood. 2004;103(3):1030-1032.

118. DiNapoli JM, Yang L, Samal SK, Murphy BR, Collins PL, Bukreyev A. Respiratory tract immunization of non-human primates with a Newcastle disease virus-vectored vaccine candidate against Ebola virus elicits a neutralizing antibody response. Vaccine. 2010;29(1):17-25.

119. Doherty PC. The tetramer transformation. J Immunol. 2011;187(1):5-6.

120. Dollenmaier G, Mosier SM, Scholle F, Sharma N, McKnight KL, Lemon SM. Membrane-associated respiratory syncytial virus F protein expressed from a human rhinovirus type 14 vector is immunogenic. Virology. 2001;281(2):216-230.

121. Dong J, Cross RW, Doyle MP, et al. Potent henipavirus neutralization by antibodies recognizing diverse sites on hendra and nipah virus receptor binding protein. Cell. 2020;183(6):1536-1550 e17.

122. Donnelly JJ, Wahren B, Liu MA. DNA vaccines: progress and challenges. J Immunol. 2005;175(2):633-639.

123. Doores KJ, Burton DR. Variable loop glycan dependency of the broad and potent HIV-1-neutralizing antibodies PG9 and PG16. J Virol. 2010;84(20):10510-10521.

124. Dowd KA, Jost CA, Durbin AP, Whitehead SS, Pierson TC. A dynamic landscape for antibody binding modulates antibody-mediated neutralization of West Nile virus. PLoS Pathog. 2011;7(6):e1002111.

125. Drexler I, Heller K, Wahren B, Erfle V, Sutter G. Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells. J Gen Virol. 1998;79: 347-352.

126. Dryden KA, Wieland SF, Whitten-Bauer C, Gerin JL, Chisari FV, Yeager M. Native hepatitis B virions and capsids visualized by electron cryomicroscopy. Mol Cell. 2006;22(6):843-850.

127. Dubin G, Socolof E, Frank I, Friedman HM. Herpes simplex virus type 1 Fc receptor protects infected cells from antibody-dependent cellular cytotoxicity. J Virol. 1991;65(12):7046-7050.

128. Dudek T, Knipe DM. Replication-defective viruses as vaccines and vaccine vectors. Virology. 2006;344(1):230-239.

129. Durst M, Gissmann L, Ikenberg H, zur Hausen H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc Natl Acad Sci U S A. 1983;80(12):3812-3815.

130. Dwyer JM, Erlendsson K. Intraventricular gamma-globulin for the management of enterovirus encephalitis. Pediatr Infect Dis J. 1988;7(5 Suppl):S30-S33.

131. Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature. 2008;453(7198):1122-1126.

132. Elliott SL, Pye S, Le T, et al. Peptide based cytotoxic T-cell vaccines; delivery of multiple epitopes, help, memory and problems. Vaccine. 1999;17(15-16):2009-2019.

133. Emu B, Fessel J, Schrader S, et al. Phase 3 study of ibalizumab for multidrug-resistant HIV-1. N Engl J Med. 2018;379(7):645-654.

134. Enelow RI, Mohammed AZ, Stoler MH, et al. Structural and functional consequences of alveolar cell recognition by CD8(+) T lymphocytes in experimental lung disease. J Clin Invest. 1998;102(9):1653-1661.

135. Ennis FA, Rook AH, Qi YH, et al. HLA restricted virus-specific cytotoxic T-lymphocyte responses to live and inactivated influenza vaccines. Lancet. 1981;2(8252):887-891.

136. Enria DA, Briggiler AM, Fernandez NJ, Levis SC, Maiztegui JI. Importance of dose of neutralising antibodies in treatment of Argentine haemorrhagic fever with immune plasma. Lancet. 1984;2(8397):255-256.

137. Fang ZY, Limbach K, Tartaglia J, Hammonds J, Chen X, Spearman P. Expression of vaccinia E3L and K3L genes by a novel recombinant canarypox HIV vaccine vector enhances HIV-1 pseudovirion production and inhibits apoptosis in human cells. Virology. 2001;291(2):272-284.

138. Felnerova D, Viret JF, Gluck R, Moser C. Liposomes and virosomes as delivery systems for antigens, nucleic acids and drugs. Curr Opin Biotechnol. 2004;15(6):518-529.

139. Fenner F, Henderson DA, Arita I, Kezek Z, Ladnyi ID. *Smallpox and Its Eradication*. World Health Organization; 1988.

140. Ferguson M, Wood DJ, Minor PD. Antigenic structure of poliovirus in inactivated vaccines. J Gen Virol. 1993;74(Pt 4):685-690.

141. Fibriansah G, Ng TS, Kostyuchenko VA, et al. Structural changes in dengue virus when exposed to a temperature of 37 degrees C. J Virol. 2013;87(13):7585-7592.

142. Flatz L, Hegazy AN, Bergthaler A, et al. Development of replication-defective lymphocytic choriomeningitis virus vectors for the induction of potent CD8+ T cell immunity. Nat Med. 2010;16(3):339-345.

143. Fleishman SJ, Whitehead TA, Ekiert DC, et al. Computational design of proteins targeting the conserved stem region of influenza hemagglutinin. Science. 2011;332(6031): 816-821.

144. Flexner C, Murphy BR, Rooney JF, et al. Successful vaccination with a polyvalent live vector despite existing immunity to an expressed antigen. Nature. 1988;335(6187):259-262.

145. Flynn BJ, Kastenmuller K, Wille-Reece U, et al. Immunization with HIV Gag targeted to dendritic cells followed by recombinant New York vaccinia virus induces robust T-cell immunity in nonhuman primates. Proc Natl Acad Sci U S A. 2011;108(17):7131-7136.

146. Flynn KJ, Belz GT, Altman JD, Ahmed R, Woodland DL, Doherty PC. Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. Immunity. 1998;8(6):683-691.

147. Flynn NM, Forthal DN, Harro CD, Judson FN, Mayer KH, Para MF. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. J Infect Dis. 2005;191(5):654-665.

148. Fouts TR, DeVico AL, Onyabe DY, et al. Progress toward the development of a bacterial vaccine vector that induces high-titer long-lived broadly neutralizing antibodies against HIV-1. FEMS Immunol Med Microbiol. 2003;37(2-3):129-134.

149. Fox J, Lennette E, Manso C, Souza-Aguiar J. Encephalitis in man following vaccination with 17D yellow fever virus. Am J Hygiene. 1941;36:117-141.

150. Fraenkel-Conrat H, Williams RC. Reconstitution of active tobacco mosaic virus from its inactive protein and nucleic acid components. Proc Natl Acad Sci U S A. 1955;41(10):690-698.

151. Franco MA, Tin C, Greenberg HB. CD8+ T cells can mediate almost complete short-term and partial long- term immunity to rotavirus in mice. J Virol. 1997;71(5):4165-4170.

152. Frey SE, Newman FK, Kennedy JS, et al. Clinical and immunologic responses to multiple doses of IMVAMUNE (Modified Vaccinia Ankara) followed by Dryvax challenge. Vaccine. 2007;25(51):8562-8573.

153. Frolov I, Hoffman TA, Pragai BM, et al. Alphavirus-based expression vectors: strategies and applications. Proc Natl Acad Sci U S A. 1996;93(21):11371-11377.

154. Fuerst TR, Earl PL, Moss B. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol Cell Biol. 1987;7(7):2538-2544.

155. Fujioka H, Emancipator SN, Aikawa M, et al. Immunocytochemical colocalization of specific immunoglobulin A with sendai virus protein in infected polarized epithelium. J Exp Med. 1998;188(7):1223-1229.

156. Fulginiti VA, Eller JJ, Downie AW, Kempe CH. Altered reactivity to measles virus. Atypical measles in children previously immunized with inactivated measles virus vaccines. JAMA. 1967;202(12):1075-1080.

157. Gans HA, Arvin AM, Galinus J, Logan L, DeHovitz R, Maldonado Y. Deficiency of the humoral immune response to measles vaccine in infants immunized at age 6 months. JAMA. 1998;280(6):527-532.

158. Gardner EM, Murasko DM. Age-related changes in Type 1 and Type 2 cytokine production in humans. Biogerontology. 2002;3(5):271-290.

159. Garland SM, Hernandez-Avila M, Wheeler CM, et al. Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases. N Engl J Med. 2007;356(19):1928-1943.

160. Georgescu MM, Balanant J, Macadam A, et al. Evolution of the Sabin type 1 poliovirus in humans: characterization of strains isolated from patients with vaccine-associated paralytic poliomyelitis. J Virol. 1997;71(10):7758-7768.

161. Gilbert PB, Peterson ML, Follmann D, et al. Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial. J Infect Dis. 2005;191(5):666-677.

162. Giuliano AR, Palefsky JM, Goldstone S, et al. Efficacy of quadrivalent HPV vaccine against HPV Infection and disease in males. N Engl J Med. 2011;364(5):401-411.

163. Glansbeek HL, Haagmans BL, te Lintelo EG, et al. Adverse effects of feline IL-12 during DNA vaccination against feline infectious peritonitis virus. J Gen Virol. 2002;83(Pt 1):1-10.

164. Glanville J, Huang H, Nau A, et al. Identifying specificity groups in the T cell receptor repertoire. Nature. 2017;547(7661):94-98.

165. Glass RI, Bresee JS, Turcios R, Fischer TK, Parashar UD, Steele AD. Rotavirus vaccines: targeting the developing world. J Infect Dis. 2005;192(Suppl 1):S160-S166.

166. Goepfert PA, Horton H, McElrath MJ, et al. High-dose recombinant canarypox vaccine expressing HIV-1 protein, in seronegative human subjects. J Infect Dis. 2005;192(7):1249-1259.

167. Gokmen E, Bachier C, Raaphorst FM, et al. Ig heavy chain CDR3 size diversities are similar after conventional peripheral blood and ex vivo expanded hematopoietic cell transplants. Bone Marrow Transplant. 2001;27(4):413-424.

168. Gorantla S, Santos K, Meyer V, et al. Human dendritic cells transduced with herpes simplex virus amplicons encoding human immunodeficiency virus type 1 (HIV-1) gp120 elicit adaptive immune responses from human cells engrafted into NOD/SCID mice and confer partial protection against HIV-1 challenge. J Virol. 2005;79(4):2124-2132.

169. Gorny MK, Williams C, Volsky B, et al. Human monoclonal antibodies specific for conformation-sensitive epitopes of V3 neutralize human immunodeficiency virus type 1 primary isolates from various clades. J Virol. 2002;76(18):9035-9045.

170. Gould SJ, Booth AM, Hildreth JE. The Trojan exosome hypothesis. Proc Natl Acad Sci U S A. 2003;100(19):10592-10597.

171. Graham BS. Biological challenges and technological opportunities for respiratory syncytial virus vaccine development. Immunol Rev. 2011;239(1):149-166.

172. Graham BS, Belshe RB, Clements ML, et al. Vaccination of vaccinia-naive adults with human immunodeficiency virus type 1 gp160 recombinant vaccinia virus in a blinded, controlled, randomized clinical trial. The AIDS Vaccine Clinical Trials Network. J Infect Dis. 1992;166(2):244-252.

173. Graham BS, Gorse GJ, Schwartz DH, et al. Determinants of antibody response after recombinant gp160 boosting in vaccinia-naive volunteers primed with gp160-recombinant vaccinia virus. The National Institute of Allergy and Infectious Diseases AIDS Vaccine Clinical Trials Network. J Infect Dis. 1994;170(4):782-786.

174. Graham BS, Henderson GS, Tang YW, Lu X, Neuzil KM, Colley DG. Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. J Immunol. 1993;151(4):2032-2040.

175. Graham BS, Keefer MC, McElrath MJ, et al. Safety and immunogenicity of a candidate HIV-1 vaccine in healthy adults: recombinant glycoprotein (rgp) 120. A randomized, double-blind trial. NIAID AIDS Vaccine Evaluation Group. Ann Intern Med. 1996;125(4):270-279.

176. Graham BS, Koup RA, Roederer M, et al. Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 DNA candidate vaccine. J Infect Dis. 2006;194(12):1650-1660.

177. Graham BS, McElrath MJ, Keefer MC, et al. Immunization with cocktail of HIV-derived peptides in montanide ISA-51 is immunogenic, but causes sterile abscesses and unacceptable reactogenicity. PLoS One. 2010;5(8):e11995.

178. Graham BS, Walker C. Meeting the challenge of vaccine design to control HIV and other difficult viruses. In: Kaufmann SHE, Rouse BT, Sacks DL, eds. The Immune Response to Infection. ASM Press; 2011:559-570.

179. Graham MB, Braciale TJ. Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice. J Exp Med. 1997;186(12):2063-2068.

180. Gray GC, Goswami PR, Malasig MD, et al. Adult adenovirus infections: loss of orphaned vaccines precipitates military respiratory disease epidemics. Clin Infect Dis. 2000;31(3):663-670.

181. Groenke N, Trimpert J, Merz S, et al. Mechanism of virus attenuation by codon pair deoptimization. Cell Rep. 2020;31(4):107586.

182. Gromeier M, Bossert B, Arita M, Nomoto A, Wimmer E. Dual stem loops within the poliovirus internal ribosomal entry site control neurovirulence. J Virol. 1999;73(2):958-964.

183. Grundner C, Li Y, Louder M, et al. Analysis of the neutralizing antibody response elicited in rabbits by repeated inoculation with trimeric HIV-1 envelope glycoproteins. Virology. 2005;331(1):33-46.

184. Guillaume JC, Saiag P, Wechsler J, Lescs MC, Roujeau JC. Vaccinia from recombinant virus expressing HIV genes. Lancet. 1991;337(8748):1034-1035.

185. Guirakhoo F, Zhang Z, Chambers TJ, et al. Immunogenicity, genetic stability, and protective efficacy of a recombinant, chimeric yellow fever-Japanese encephalitis virus (ChimeriVax-JE) as a live, attenuated vaccine candidate against Japanese encephalitis. Virology. 1999;257(2):363-372.

186. Gutekunst RR, White RJ, Edmondson WP, Chanock RM. Immunization with live type 4 adenovirus: determination of infectious virus dose and protective effect of enteric infection. Am J Epidemiol. 1967;86(2):341-349.

187. Guthmiller JJ, Han J, Utset HA, et al. Broadly neutralizing antibodies target a haemagglutinin anchor epitope. Nature. 2022;602(7896):314-320.

188. Guy B, Guirakhoo F, Barban V, Higgs S, Monath TP, Lang J. Preclinical and clinical development of YFV 17D-based chimeric vaccines against dengue, West Nile and Japanese encephalitis viruses. Vaccine. 2010;28(3):632-649.

189. Ha SJ, Mueller SN, Wherry EJ, et al. Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection. J Exp Med. 2008;205(3): 543-555.

190. Hacein-Bey-Abina S, von Kalle C, Schmidt M, et al. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med. 2003;348(3):255-256.

191. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science. 2003;302(5644):415-419.

192. Haffar O, Garrigues J, Travis B, Moran P, Zarling J, Hu SL. Human immunodeficiency virus-like, nonreplicating, gag-env particles

assemble in a recombinant vaccinia virus expression system. J Virol. 1990;64(6):2653-2659.

193. Haglund K, Leiner I, Kerksiek K, Buonocore L, Pamer E, Rose JK. High-level primary CD8(+) T-cell response to human immunodeficiency virus type 1 gag and env generated by vaccination with recombinant vesicular stomatitis viruses. J Virol. 2002;76(6):2730-2738.

194. Halstead SB. Pathogenesis of dengue: challenges to molecular biology. Science. 1988;239(4839):476-481.

195. Hammitt LL, Dagan R, Yuan Y, et al. Nirsevimab for prevention of RSV in healthy late-preterm and term infants. N Engl J Med. 2022;386(9):837-846.

196. Hammon WM, Coriell LL, Wehrle PF, Stokes J Jr. Evaluation of Red Cross gamma globulin as a prophylactic agent for poliomyelitis. IV. Final report of results based on clinical diagnoses. J Am Med Assoc. 1953;151(15):1272-1285.

197. Hammonds J, Chen X, Zhang X, Lee F, Spearman P. Advances in methods for the production, purification, and characterization of HIV-1 Gag-Env pseudovirion vaccines. Vaccine. 2007;25(47):8036-8048.

198. Hanke T, Blanchard TJ, Schneider J, et al. Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime. Vaccine. 1998;16(5):439-445.

199. Hanna SL, Pierson TC, Sanchez MD, Ahmed AA, Murtadha MM, Doms RW. N-linked glycosylation of West Nile virus envelope proteins influences particle assembly and infectivity. J Virol. 2005;79(21):13262-13274.

200. Hansen SG, Ford JC, Lewis MS, et al. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. Nature. 2011;473(7348):523-527.

201. Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol. 2005;6(11):1123-1132.

202. Harrington PR, Yount B, Johnston RE, Davis N, Moe C, Baric RS. Systemic, mucosal, and heterotypic immune induction in mice inoculated with Venezuelan equine encephalitis replicons expressing Norwalk virus-like particles. J Virol. 2002;76(2):730-742.

203. Hart TK, Kirsh R, Ellens H, et al. Binding of soluble CD4 proteins to human immunodeficiency virus type 1 and infected cells induces release of envelope glycoprotein gp120. Proc Natl Acad Sci U S A. 1991;88(6):2189-2193.

204. Hasenkrug KJ, Brooks DM, Dittmer U. Critical role for CD4(+) T cells in controlling retrovirus replication and spread in persistently infected mice. J Virol. 1998;72(8):6559-6564.

205. Hawiger J. Noninvasive intracellular delivery of functional peptides and proteins. Curr Opin Chem Biol. 1999;3(1):89-94.

206. Haynes BF, Fleming J, St Clair EW, et al. Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. Science. 2005;308(5730):1906-1908.

207. Hessell AJ, Hangartner L, Hunter M, et al. Fc receptor but not complement binding is important in antibody protection against HIV. Nature. 2007;449(7158):101-104.

208. Hevey M, Negley D, Pushko P, Smith J, Schmaljohn A. Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. Virology. 1998;251(1):28-37.

209. Hodgson LA, Ludwig GV, Smith JF. Expression, processing, and immunogenicity of the structural proteins of Venezuelan equine encephalitis virus from recombinant baculovirus vectors. Vaccine. 1999;17(9-10):1151-1160.

210. Holt EA, Moulton LH, Siberry GK, Halsey NA. Differential mortality by measles vaccine titer and sex. J Infect Dis. 1993;168(5):1087-1096.

211. Horvath B, Fornosi F. Excretion of SV40 virus after oral administration of contaminated polio vaccine. Acta Microbiol Hung. 1964;11:271-275.

212. Hsu HM, Lu CF, Lee SC, Lin SR, Chen DS. Seroepidemiologic survey for hepatitis B virus infection in Taiwan: the effect of hepatitis B mass immunization. J Infect Dis. 1999;179(2):367-370.

213. Huang Y, Yang ZY, Kong WP, Nabel GJ. Generation of synthetic severe acute respiratory syndrome coronavirus pseudoparticles: implications for assembly and vaccine production. J Virol. 2004;78(22):12557-12565.

214. Hurwitz JL. Development of recombinant Sendai virus vaccines for prevention of human parainfluenza and respiratory syncytial virus infections. Pediatr Infect Dis J. 2008;27(10 Suppl):S126-S128.

215. Hussey GD, Goddard EA, Hughes J, et al. The effect of Edmonston-Zagreb and Schwarz measles vaccines on immune response in infants. J Infect Dis. 1996;173(6):1320-1326.

216. Ikizler MR, Wright PF. Thermostabilization of egg grown influenza viruses. Vaccine. 2002;20(9-10):1393-1399.

217. Issel CJ, Horohov DW, Lea DF, et al. Efficacy of inactivated whole-virus and subunit vaccines in preventing infection and disease caused by equine infectious anemia virus. J Virol. 1992;66(6):3398-3408.

218. Janetzki S, Panageas KS, Ben-Porat L, et al. Results and harmonization guidelines from two large-scale international Elispot proficiency panels conducted by the Cancer Vaccine Consortium (CVC/SVI). Cancer Immunol Immunother. 2008;57(3):303-315.

219. Janeway CA. Use of concentrated human gamma globulin in the prevention and attenuation of measles. Bull NY Acad Med. 1945;21:202-202.

220. Johnson PR, Feldman S, Thompson JM, Mahoney JD, Wright PF. Immunity to influenza A virus infection in young children: a comparison of natural infection, live cold-adapted vaccine, and inactivated vaccine. J Infect Dis. 1986;154(1):121-127.

221. Johnson PR, Schnepp BC, Zhang J, et al. Vector-mediated gene transfer engenders long-lived neutralizing activity and protection against SIV infection in monkeys. Nat Med. 2009;15(8):901-906.

222. Johnston RE, Johnson PR, Connell MJ, et al. Vaccination of macaques with SIV immunogens delivered by Venezuelan equine encephalitis virus replicon particle vectors followed by a mucosal challenge with SIVsmE660. Vaccine. 2005;23(42):4969-4979.

223. Jones D, Kroos N, Anema R, et al. High-level expression of recombinant IgG in the human cell line per.c6. Biotechnol Prog. 2003;19(1):163-168.

224. Jumper J, Evans R, Pritzel A, et al. Highly accurate protein structure prediction with AlphaFold. Nature. 2021;596(7873):583-589.

225. Kaech SM, Hemby S, Kersh E, Ahmed R. Molecular and functional profiling of memory CD8 T cell differentiation. Cell. 2002;111(6):837-851.

226. Kalinke U, Bucher EM, Ernst B, et al. The role of somatic mutation in the generation of the protective humoral immune response against vesicular stomatitis virus. Immunity. 1996;5(6):639-652.

227. Kallewaard NL, McKinney BA, Gu Y, Chen A, Prasad BV, Crowe JE Jr. Functional maturation of the human antibody response to rotavirus. J Immunol. 2008;180(6):3980-3989.

228. Kanekiyo M, Joyce MG, Gillespie RA, et al. Mosaic nanoparticle display of diverse influenza virus hemagglutinins elicits broad B cell responses. Nat Immunol. 2019;20(3):362-372.

229. Kapikian AZ, Simonsen L, Vesikari T, et al. A hexavalent human rotavirus-bovine rotavirus (UK) reassortant vaccine designed for use in developing countries and delivered in a schedule with the potential to eliminate the risk of intussusception. J Infect Dis. 2005;192(Suppl 1):S22-S29.

230. Karron RA, Belshe RB, Wright PF, et al. A live human parainfluenza type 3 virus vaccine is attenuated and immunogenic in young infants. Pediatr Infect Dis J. 2003;22(5):394-405.

231. Karron RA, Wright PF, Belshe RB, et al. Identification of a recombinant live attenuated respiratory syncytial virus vaccine candidate that is highly attenuated in infants. J Infect Dis. 2005;191(7):1093-1104.

232. Kast WM, Roux L, Curren J, et al. Protection against lethal Sendai virus infection by in vivo priming of virus-specific cytotoxic T lymphocytes with a free synthetic peptide. Proc Natl Acad Sci U S A. 1991;88(6):2283-2287.

233. Kenter GG, Welters MJ, Valentijn AR, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. N Engl J Med. 2009;361(19):1838-1847.

234. Khurana S, Verma N, Yewdell JW, et al. MF59 adjuvant enhances diversity and affinity of antibody-mediated immune response to pandemic influenza vaccines. Sci Transl Med. 2011;3(85):85ra48.

235. Kim HW, Canchola JG, Brandt CD, et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol. 1969;89(4):422-434.

236. Kim SK, Reed DS, Olson S, et al. Generation of mucosal cytotoxic T cells against soluble protein by tissue-specific environmental and costimulatory signals. Proc Natl Acad Sci U S A. 1998;95(18):10814-10819.

237. Kim YC, Jarrahian C, Zehrung D, Mitragotri S, Prausnitz MR. Delivery systems for intradermal vaccination. Curr Top Microbiol Immunol. 2012;351:77-112.

238. Kines RC, Thompson CD, Lowy DR, Schiller JT, Day PM. The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. Proc Natl Acad Sci U S A. 2009;106(48):20458-20463.

239. Kirnbauer R, Booy F, Cheng N, Lowy DR, Schiller JT. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proc Natl Acad Sci U S A. 1992;89(24):12180-12184.

240. Kobayashi T, Antar AA, Boehme KW, et al. A plasmid-based reverse genetics system for animal double-stranded RNA viruses. Cell Host Microbe. 2007;1(2):147-157.

241. Kohl S. Role of antibody-dependent cellular cytotoxicity in defense against herpes simplex virus infections. Rev Infect Dis. 1991;13(1):108-114.

242. Kollmann TR, Marchant A, Way SS. Vaccination strategies to enhance immunity in neonates. Science. 2020;368(6491):612-615.

243. Kong R, Xu K, Zhou T, et al. Fusion peptide of HIV-1 as a site of vulnerability to neutralizing antibody. Science. 2016;352(6287):828-833.

244. Konopka JL, Thompson JM, Whitmore AC, Webb DL, Johnston RE. Acute infection with venezuelan equine encephalitis virus replicon particles catalyzes a systemic antiviral state and protects from lethal virus challenge. J Virol. 2009;83(23):12432-12442.

245. Kostense S, Koudstaal W, Sprangers M, et al. Adenovirus types 5 and 35 seroprevalence in AIDS risk groups supports type 35 as a vaccine vector. AIDS. 2004;18(8):1213-1216.

246. Kostense S, Raaphorst FM, Notermans DW, et al. Diversity of the T-cell receptor BV repertoire in HIV-1-infected patients reflects the biphasic CD4+ T-cell repopulation kinetics during highly active antiretroviral therapy. AIDS. 1998;12(18):F235-F240.

247. Koup RA, Roederer M, Lamoreaux L, et al. Priming immunization with DNA augments immunogenicity of recombinant adenoviral vectors for both HIV-1 specific antibody and T-cell responses. PLoS One. 2010;5(2):e9015.

248. Kouri G, Guzman MG, Valdes L, et al. Reemergence of dengue in Cuba: a 1997 epidemic in Santiago de Cuba. Emerg Infect Dis. 1998;4(1):89-92.

249. Koutsky LA, Ault KA, Wheeler CM, et al. A controlled trial of a human papillomavirus type 16 vaccine. N Engl J Med. 2002;347(21):1645-1651.

250. Krause JC, Tumpey TM, Huffman CJ, et al. Naturally occurring human monoclonal antibodies neutralize both 1918 and 2009 pandemic influenza A (H1N1) viruses. J Virol. 2010;84(6):3127-3130.

251. Kundig TM, Castelmur I, Bachmann MF, et al. Fewer protective cytotoxic T-cell epitopes than T-helper-cell epitopes on vesicular stomatitis virus. J Virol. 1993;67(6):3680-3683.

252. Kwakkenbos MJ, Diehl SA, Yasuda E, et al. Generation of stable monoclonal antibody-producing B cell receptor-positive human memory B cells by genetic programming. Nat Med. 2010;16(1):123-128.

253. Kwong PD, Doyle ML, Casper DJ, et al. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. Nature. 2002;420(6916): 678-682.

254. Kwong PD, Wilson IA. HIV-1 and influenza antibodies: seeing antigens in new ways. Nat Immunol. 2009;10(6):573-578.

255. Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature. 1998;393(6686):648-659.

256. Lamparski HG, Metha-Damani A, Yao JY, et al. Production and characterization of clinical grade exosomes derived from dendritic cells. J Immunol Methods. 2002;270(2):211-226.

257. Langbeheim H, Arnon R, Sela M. Antiviral effect on MS-2 coliphage obtained with a synthetic antigen. Proc Natl Acad Sci U S A. 1976;73(12):4636-4640.

258. Lasky T, Terracciano GJ, Magder L, et al. The Guillain-Barre syndrome and the 1992-1993 and 1993-1994 influenza vaccines. N Engl J Med. 1998;339(25):1797-1802.

259. Latham T, Galarza JM. Formation of wild-type and chimeric influenza virus-like particles following simultaneous expression of only four structural proteins. J Virol. 2001;75(13):6154-6165.

260. Lau YL, Fan Leung T, Sirvan Cetin B, et al. Safety and reactogenicity of a liquid formulation of human rotavirus vaccine (porcine circovirus-free): a phase III, observer-blind, randomized, multi-country study. Vaccine. 2022;40(14):2184-2190.

261. Ledgerwood JE, Pierson TC, Hubka SA, et al. A West Nile virus DNA vaccine utilizing a modified promoter induces neutralizing antibody in younger and older healthy adults in a phase I clinical trial. J Infect Dis. 2011;203(10):1396-1404.

262. Lehner T, Bergmeier LA, Tao L, et al. Targeted lymph node immunization with simian immunodeficiency virus p27 antigen to elicit genital, rectal, and urinary immune responses in nonhuman primates. J Immunol. 1994;153(4):1858-1868.

263. Lemiale F, Kong WP, Akyurek LM, et al. Enhanced mucosal immunoglobulin A response of intranasal adenoviral vector human immunodeficiency virus vaccine and localization in the central nervous system. J Virol. 2003;77(18):10078-10087.

264. Lennon RG, Isacson P. Delayed dermal hypersensitivity following killed measles vaccine. Experience in 9-month-old infants. J Pediatr. 1967;71(4):525-529.

265. Letvin NL, Rao SS, Montefiori DC, et al. Immune and genetic correlates of vaccine protection against mucosal infection by SIV in monkeys. Sci Transl Med. 2011;3(81):81ra36.

266. Levine B, Hardwick JM, Trapp BD, Crawford TO, Bollinger RC, Griffin DE. Antibody-mediated clearance of alphavirus infection from neurons. Science. 1991;254(5033):856-860.

267. Lewis GK, Ackerman ME, Scarlatti G, et al. Knowns and unknowns of assaying antibody-dependent cell-mediated cytotoxicity against HIV-1. Front Immunol. 2019;10:1025.

268. Liao HX, Cianciolo GJ, Staats HF, et al. Increased immunogenicity of HIV envelope subunit complexed with alpha2-macroglobulin when combined with monophosphoryl lipid A and GM-CSF. Vaccine. 2002;20(17-18):2396-2403.

269. Little SR, Lynn DM, Ge Q, et al. Poly-beta amino ester-containing microparticles enhance the activity of nonviral genetic vaccines. Proc Natl Acad Sci U S A. 2004;101(26):9534-9539.

270. Locher CP, Putnam D, Langer R, Witt SA, Ashlock BM, Levy JA. Enhancement of a human immunodeficiency virus env DNA vaccine using a novel polycationic nanoparticle formulation. Immunol Lett. 2003;90(2-3):67-70.

271. Lowe RS, Brown DR, Bryan JT, et al. Human papillomavirus type 11 (HPV-11) neutralizing antibodies in the serum and genital mucosal secretions of African green monkeys immunized with HPV-11 virus-like particles expressed in yeast. J Infect Dis. 1997;176(5):1141-1145.

272. Lu W, Arraes LC, Ferreira WT, Andrieu JM. Therapeutic dendritic-cell vaccine for chronic HIV-1 infection. Nat Med. 2004;10(12):1359-1365.

273. Lubeck MD, Natuk R, Myagkikh M, et al. Long-term protection of chimpanzees against high-dose HIV-1 challenge induced by

immunization. Nat Med. 1997;3(6):651-658.

274. Luckow V, Summers M. Trends in the development of baculovirus expression vectors. Biotechnology. 1988;6:47-55.

275. Lusky M. Good manufacturing practice production of adenoviral vectors for clinical trials. Hum Gene Ther. 2005;16(3):281-291.

276. MacDonald GH, Johnston RE. Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis. J Virol. 2000;74(2):914-922.

277. Mackett M, Archard LC. Conservation and variation in Orthopoxvirus genome structure. J Gen Virol. 1979;45(3):683-701.

278. Maecker HT, Hassler J, Payne JK, et al. Precision and linearity targets for validation of an IFNgamma ELISPOT, cytokine flow cytometry, and tetramer assay using CMV peptides. BMC Immunol. 2008;9:9.

279. Maiztegui JI, Fernandez NJ, de Damilano AJ. Efficacy of immune plasma in treatment of Argentine haemorrhagic fever and association between treatment and a late neurological syndrome. Lancet. 1979;2(8154):1216-1217.

280. Manickan E, Francotte M, Kuklin N, et al. Vaccination with recombinant vaccinia viruses expressing ICP27 induces protective immunity against herpes simplex virus through CD4+ Th1+ T cells. J Virol. 1995;69(8):4711-4716.

281. Marovich MA, Mascola JR, Eller MA, et al. Preparation of clinical-grade recombinant canarypox-human immunodeficiency virus vaccine-loaded human dendritic cells. J Infect Dis. 2002;186(9):1242-1252.

282. Martin JE, Pierson TC, Hubka S, et al. A West Nile virus DNA vaccine induces neutralizing antibody in healthy adults during a phase 1 clinical trial. J Infect Dis. 2007;196(12):1732-1740.

283. Mascola JR. Passive transfer studies to elucidate the role of antibody-mediated protection against HIV-1. Vaccine. 2002;20(15):1922-1925.

284. Mayr A, Hochstein-Mintzel V, Stickl H. Passage history, properties, and applicability of the attenuated vaccinia virus strain MVA. Infection. 1975;3:6-14.

285. Mayr A, Stickl H, Muller HK, Danner K, Singer H. The smallpox vaccination strain MVA: marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism (author's transl). Zentralbl Bakteriol [B]. 1978;167(5-6):375-390.

286. Mazanec MB, Kaetzel CS, Lamm ME, Fletcher D, Nedrud JG. Intracellular neutralization of virus by immunoglobulin A antibodies. Proc Natl Acad Sci U S A. 1992;89(15):6901-6905.

287. Mazanec MB, Kaetzel CS, Lamm ME, Fletcher D, Peterra J, Nedrud JG. Intracellular neutralization of Sendai and influenza viruses by IgA monoclonal antibodies. Adv Exp Med Biol. 1995;371A:651-654.

288. McClain DJ, Pittman PR, Ramsburg HH, et al. Immunologic interference from sequential administration of live attenuated alphavirus vaccines. J Infect Dis. 1998;177(3):634-641.

289. McCurdy LH, Rutigliano JA, Johnson TR, Chen M, Graham BS. Modified vaccinia virus Ankara immunization protects against lethal challenge with recombinant vaccinia virus expressing murine interleukin-4. J Virol. 2004;78(22):12471-12479.

290. McGinnes LW, Gravel KA, Finberg RW, et al. Assembly and immunological properties of Newcastle disease virus-like particles containing the respiratory syncytial virus F and G proteins. J Virol. 2011;85(1):366-377.

291. McGuire TC, Adams DS, Johnson GC, Klevjer-Anderson P, Barbee DD, Gorham JR. Acute arthritis in caprine arthritis-encephalitis virus challenge exposure of vaccinated or persistently infected goats. Am J Vet Res. 1986;47(3):537-540.

292. McLellan JS, Correia BE, Chen M, et al. Design and characterization of epitope-scaffold immunogens that present the motavizumab epitope from respiratory syncytial virus. J Mol Biol. 2011;409(5):853-866.

293. Mease PJ, Ochs HD, Wedgwood RJ. Successful treatment of echovirus meningoencephalitis and myositis-fasciitis with intravenous immune globulin therapy in a patient with X-linked agammaglobulinemia. N Engl J Med. 1981;304(21):1278-1281.

294. Men R, Bray M, Clark D, Chanock RM, Lai CJ. Dengue type 4 virus mutants containing deletions in the 3′ noncoding region of the RNA genome: analysis of growth restriction in cell culture and altered viremia pattern and immunogenicity in rhesus monkeys. J Virol. 1996;70(6):3930-3937.

295. Mercadal CM, Bouley DM, DeStephano D, Rouse BT. Herpetic stromal keratitis in the reconstituted scid mouse model. J Virol. 1993;67(6):3404-3408.

296. Meyer H, Sutter G, Mayr A. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. J Gen Virol. 1991;72: 1031-1038.

297. Meyer HM Jr, Hopps HE, Parkman PD, Ennis FA. Control of measles and rubella through use of attenuated vaccines. Am J Clin Pathol. 1978;70(1 Suppl):128-135.

298. Mezhenskaya D, Isakova-Sivak I, Rudenko L. M2e-based universal influenza vaccines: a historical overview and new approaches to development. J Biomed Sci. 2019;26(1):76.

299. Mikszta JA, Alarcon JB, Brittingham JM, Sutter DE, Pettis RJ, Harvey NG. Improved genetic immunization via micromechanical disruption of skin-barrier function and targeted epidermal delivery. Nat Med. 2002;8(4):415-419.

300. Milstien JB, Lemon SM, Wright PF. Development of a more thermostable poliovirus vaccine. J Infect Dis. 1997;175(Suppl 1):S247-S253.

301. Modlin JF, Halsey NA, Thoms ML, Meschievitz CK, Patriarca PA. Humoral and mucosal immunity in infants induced by three sequential inactivated poliovirus vaccine-live attenuated oral poliovirus vaccine immunization schedules. Baltimore Area Polio Vaccine Study Group. J Infect Dis. 1997;175(Suppl 1):S228-S234.

302. Moghaddam A, Olszewska W, Wang B, et al. A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines. Nat Med. 2006;12(8):905-907.

303. Monath TP, Guirakhoo F, Nichols R, et al. Chimeric live, attenuated vaccine against Japanese encephalitis (ChimeriVax-JE): phase 2 clinical trials for safety and immunogenicity, effect of vaccine dose and schedule, and memory response to challenge with inactivated Japanese encephalitis antigen. J Infect Dis. 2003;188(8):1213-1230.

304. Monsalvo AC, Batalle JP, Lopez MF, et al. Severe pandemic 2009 H1N1 influenza disease due to pathogenic immune complexes. Nat Med. 2011;17(2):195-199.

305. Montgomery DL, Shiver JW, Leander KR, et al. Heterologous and homologous protection against influenza A by DNA vaccination: optimization of DNA vectors. DNA Cell Biol. 1993;12(9):777-783.

306. Morrison LA, Da Costa XJ, Knipe DM. Influence of mucosal and parenteral immunization with a replication- defective mutant of HSV-2 on immune responses and protection from genital challenge. Virology. 1998;243(1):178-187.

307. Morrison TG. Newcastle disease virus-like particles as a platform for the development of vaccines for human and agricultural pathogens. Future Virol. 2010;5(5):545-554.

308. Mortimer EA Jr, Lepow ML, Gold E, Robbins FC, Burton GJ, Fraumeni JF Jr. Long-term follow-up of persons inadvertently inoculated with SV40 as neonates. N Engl J Med. 1981;305(25):1517-1518.

309. Moser C, Metcalfe IC, Viret JF. Virosomal adjuvanted antigen delivery systems. Expert Rev Vaccines. 2003;2(2):189-196.

310. Moskophidis D, Kioussis D. Contribution of virus-specific CD8+ cytotoxic T cells to virus clearance or pathologic manifestations of influenza virus infection in a T cell receptor transgenic mouse model. J Exp Med. 1998;188(2):223-232.

311. Moss B, Flexner C. Vaccinia virus expression vectors. Annu Rev Immunol. 1987;5:305-324.

312. Mouquet H, Scheid JF, Zoller MJ, et al. Polyreactivity increases the apparent affinity of anti-HIV antibodies by heteroligation. Nature. 2010;467(7315):591-595.

313. Mozdzanowska K, Furchner M, Maiese K, Gerhard W. CD4+ T cells are ineffective in clearing a pulmonary infection with influenza type A virus in the absence of B cells. Virology. 1997;239(1):217-225.

314. Mozdzanowska K, Maiese K, Furchner M, Gerhard W. Treatment of influenza virus-infected SCID mice with nonneutralizing antibodies specific for the transmembrane proteins matrix 2 and neuraminidase reduces the pulmonary virus titer but fails to clear the infection. Virology. 1999;254(1):138-146.

315. Mueller S, Wimmer E. Introducing recombinant picornaviral genomes into cells. Cold Spring Harb Protoc. 2011;2011(6):657-663.

316. Mulangu S, Dodd LE, Davey RT Jr, et al. A randomized, controlled trial of Ebola virus disease therapeutics. N Engl J Med. 2019;381(24):2293-2303.

317. Mullbacher A, Ebnet K, Blanden RV, et al. Granzyme A is critical for recovery of mice from infection with the natural cytopathic viral pathogen, ectromelia. Proc Natl Acad Sci U S A. 1996;93(12):5783-5787.

318. Mumper RJ, Cui Z. Genetic immunization by jet injection of targeted pDNA-coated nanoparticles. Methods. 2003;31(3):255-262.

319. Murphy BR. Mucosal immunity to viruses. In: Ogra PL, Mestecky J, Lamm ME, Strober W, McGhee JR, Bienstock J, eds. Mucosal Immunology. 2nd ed. Academic Press, Inc.; 1999:695-707.

320. Murphy BR, Morens DM, Simonsen L, Chanock RM, La Montagne JR, Kapikian AZ. Reappraisal of the association of intussusception with the licensed live rotavirus vaccine challenges initial conclusions. J Infect Dis. 2003;187(8):1301-1308.

321. Murphy BR, Park EJ, Gottlieb P, Subbarao K. An influenza A live attenuated reassortant virus possessing three temperature-sensitive mutations in the PB2 polymerase gene rapidly loses temperature sensitivity following replication in hamsters. Vaccine. 1997;15(12-13):1372-1378.

322. Murphy BR, Prince GA, Walsh EE, et al. Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. J Clin Microbiol. 1986;24(2):197-202.

323. Murphy BR, Sotnikov A, Paradiso PR, et al. Immunization of cotton rats with the fusion (F) and large (G) glycoproteins of respiratory syncytial virus (RSV) protects against RSV challenge without potentiating RSV disease. Vaccine. 1989;7(6):533-540.

324. Murphy BR, Whitehead SS. Immune response to dengue virus and prospects for a vaccine. Annu Rev Immunol. 2011;29:587-619.

325. Murphy CG, Lucas WT, Means RE, et al. Vaccine protection against simian immunodeficiency virus by recombinant strains of herpes simplex virus. J Virol. 2000;74(17): 7745-7754.

326. Murray PJ, Aldovini A, Young RA. Manipulation and potentiation of antimycobacterial immunity using recombinant bacille Calmette-Guerin strains that secrete cytokines. Proc Natl Acad Sci U S A. 1996;93(2):934-939.

327. Nader P, Horwitz M, Rousseau J. Atypical exanthem following exposure to natural measles. Eleven cases in children previously inoculated with killed vaccine. J Pediatr. 1968;72:22-28.

328. Nathanson N, Langmuir AD. The Cutter incident. Poliomyelitis following formaldehyde-inactivated poliovirus vaccination in the United States during the Spring of 1955. II. Relationship of poliomyelitis to Cutter vaccine. 1963 [classical article]. Am J Epidemiol. 1995;142(2):109-140; discussion 107-108.

329. Nchinda G, Kuroiwa J, Oks M, et al. The efficacy of DNA vaccination is enhanced in mice by targeting the encoded protein to dendritic cells. J Clin Invest. 2008;118(4):1427-1436.

330. Neuzil KM, Johnson JE, Tang YW, et al. Adjuvants influence the quantitative and qualitative immune response in BALB/c mice immunized with respiratory syncytial virus FG subunit vaccine. Vaccine. 1997;15(5):525-532.

331. Nicholson KG, Abrams KR, Batham S, et al. Immunogenicity and safety of a two-dose schedule of whole-virion and AS03A-adjuvanted 2009 influenza A (H1N1) vaccines: a randomised, multicentre, age-stratified, head-to-head trial. Lancet Infect Dis. 2011;11(2):91-101.

332. Niikura M, Takamura S, Kim G, et al. Chimeric recombinant hepatitis E virus-like particles as an oral vaccine vehicle presenting foreign epitopes. Virology. 2002;293(2):273-280.

333. Nishimura Y, Gautam R, Chun TW, et al. Early antibody therapy can induce long-lasting immunity to SHIV. Nature. 2017;543(7646):559-563.

334. Nkowane BM, Wassilak SG, Orenstein WA, et al. Vaccine-associated paralytic poliomyelitis. United States: 1973 through 1984. JAMA. 1987;257(10):1335-1340.

335. Nooraei S, Bahrulolum H, Hoseini ZS, et al. Virus-like particles: preparation, immunogenicity and their roles as nanovaccines and drug nanocarriers. J Nanobiotechnology. 2021;19(1):59.

336. Norrby E, Enders-Ruckle G, Meulen V. Differences in the appearance of antibodies to structural components of measles virus after immunization with inactivated and live virus. J Infect Dis. 1975;132(3):262-269.

337. Norrby E, Gollmar Y. Identification of measles virus-specific hemolysis-inhibiting antibodies separate from hemagglutination-inhibiting antibodies. Infect Immun. 1975;11(2):231-239.

338. Norris PJ, Moffett HF, Yang OO, et al. Beyond help: direct effector functions of human immunodeficiency virus type 1-specific CD4(+) T cells. J Virol. 2004;78(16):8844-8851.

339. Norris PJ, Sumaroka M, Brander C, et al. Multiple effector functions mediated by human immunodeficiency virus-specific CD4(+) T-cell clones. J Virol. 2001;75(20):9771-9779.

340. Ofek G, Guenaga FJ, Schief WR, et al. Elicitation of structure-specific antibodies by epitope scaffolds. Proc Natl Acad Sci U S A. 2010;107(42):17880-17887.

341. Offit PA. The Cutter Incident: How America's First Polio Vaccine Led to the Growing Vaccine Crisis. Yale University Press; 2007.

342. O'Hagan DT, Singh M, Ulmer JB. Microparticles for the delivery of DNA vaccines. Immunol Rev. 2004;199:191-200.

343. Oi VT, Vuong TM, Hardy R, et al. Correlation between segmental flexibility and effector function of antibodies. Nature. 1984;307(5947):136-140.

344. O'Neill D, Bhardwaj N. Generation of autologous peptide- and protein-pulsed dendritic cells for patient-specific immunotherapy. Methods Mol Med. 2005;109:97-112.

345. Orentas RJ, Hildreth JE, Obah E, et al. Induction of CD4+ human cytolytic T cells specific for HIV-infected cells by a gp160 subunit vaccine. Science. 1990;248(4960):1234-1237.

346. Osborne JD, Da Silva M, Frace AM, et al. Genomic differences of Vaccinia virus clones from Dryvax smallpox vaccine: the Dryvax-like ACAM2000 and the mouse neurovirulent Clone-3. Vaccine. 2007;25(52):8807-8832.

347. Outlaw MC, Armstrong SJ, Dimmock NJ. Mechanisms of neutralization of influenza virus in tracheal epithelial and BHK cells vary according to IgG concentration. Virology. 1990;178(2):478-485.

348. Oxman MN, Levin MJ, Johnson GR, et al. A vaccine to prevent herpes zoster and postherpetic neuralgia in older adults. N Engl J Med. 2005;352(22):2271-2284.

349. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. The IMpact-RSV Study Group. Pediatrics. 1998;102(3 Pt 1):531-537.

350. Palladino G, Mozdzanowska K, Washko G, Gerhard W. Virus-neutralizing antibodies of immunoglobulin G (IgG) but not of IgM or IgA isotypes can cure influenza virus pneumonia in SCID mice. J Virol. 1995;69(4):2075-2081.

351. Palmenberg AC. In vitro synthesis and assembly of picornaviral capsid intermediate structures. J Virol. 1982;44(3):900-906.

352. Pancera M, Wyatt R. Selective recognition of oligomeric HIV-1 primary isolate envelope glycoproteins by potently neutralizing ligands requires efficient precursor cleavage. Virology. 2005;332(1):145-156.

353. Pantua HD, McGinnes LW, Peeples ME, Morrison TG. Requirements for the assembly and release of Newcastle disease virus-like particles. J Virol. 2006;80(22):11062-11073.

354. Paoletti E. Applications of pox virus vectors to vaccination: an update. Proc Natl Acad Sci U S A. 1996;93(21):11349-11353.

355. Parkman PD. Making vaccination policy: the experience with rubella. Clin Infect Dis. 1999;28(Suppl 2):S140-S146.

356. Parr EL, Parr MB. Immunoglobulin G, plasma cells, and lymphocytes in the murine vagina after vaginal or parenteral immunization with attenuated herpes simplex virus type 2. J Virol. 1998;72(6):5137-5145.

357. Parrino J, McCurdy LH, Larkin BD, et al. Safety, immunogenicity and efficacy of modified vaccinia Ankara (MVA) against Dryvax challenge in vaccinia-naive and vaccinia-immune individuals. Vaccine. 2007;25(8):1513-1525.

358. Pastoret PP, Brochier B. Epidemiology and control of fox rabies in Europe. Vaccine. 1999;17(13-14):1750-1754.

359. Pawelec G, Akbar A, Caruso C, Solana R, Grubeck-Loebenstein B, Wikby A. Human immunosenescence: is it infectious? Immunol Rev. 2005;205:257-268.

360. Pedersen NC, Black JW. Attempted immunization of cats against feline infectious peritonitis, using avirulent live virus or sublethal amounts of virulent virus. Am J Vet Res. 1983;44(2):229-234.

361. Pejchal R, Walker LM, Stanfield RL, et al. Structure and function of broadly reactive antibody PG16 reveal an H3 subdomain that mediates potent neutralization of HIV-1. Proc Natl Acad Sci U S A. 2010;107(25):11483-11488.

362. Peng B, Wang LR, Gomez-Roman VR, et al. Replicating rather than nonreplicating adenovirus-human immunodeficiency virus recombinant vaccines are better at eliciting potent cellular immunity and priming high-titer antibodies. J Virol. 2005;79(16):10200-10209.

363. Perfetto SP, Chattopadhyay PK, Roederer M. Seventeen-colour flow cytometry: unravelling the immune system. Nat Rev Immunol. 2004;4(8):648-655.

364. Phung E, Chang LA, Mukhamedova M, et al. Elicitation of pneumovirus-specific B cell responses by a prefusion-stabilized respiratory syncytial virus F subunit vaccine. Sci Transl Med. 2022;14(650):eabo5032.

365. Pihlgren M, Schallert N, Tougne C, et al. Delayed and deficient establishment of the long-term bone marrow plasma cell pool during early life. Eur J Immunol. 2001;31(3):939-946.

366. Piirainen L, Stenvik M, Roivainen M, Eskola J, Beuvery EC, Hovi T. Randomised, controlled trial with the trypsin-modified inactivated poliovirus vaccine: assessment of intestinal immunity with live challenge virus. Vaccine. 1999;17(9-10):1084-1090.

367. Pimentel TA, Yan Z, Jeffers SA, Holmes KV, Hodges RS, Burkhard P. Peptide nanoparticles as novel immunogens: design and analysis of a prototypic severe acute respiratory syndrome vaccine. Chem Biol Drug Des. 2009;73(1):53-61.

368. Pitisuttithum P, Gilbert P, Gurwith M, et al. Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. J Infect Dis. 2006;194(12):1661-1671.

369. Pitisuttithum P, Nitayaphan S, Thongcharoen P, et al. Safety and immunogenicity of combinations of recombinant subtype E and B human immunodeficiency virus type 1 envelope glycoprotein 120 vaccines in healthy Thai adults. J Infect Dis. 2003;188(2):219-227.

370. Pittman PR, Makuch RS, Mangiafico JA, Cannon TL, Gibbs PH, Peters CJ. Long-term duration of detectable neutralizing antibodies after administration of live-attenuated VEE vaccine and following booster vaccination with inactivated VEE vaccine. Vaccine. 1996;14(4):337-343.

371. Pletnev AG, Men R. Attenuation of the Langat tick-borne flavivirus by chimerization with mosquito-borne flavivirus dengue type 4. Proc Natl Acad Sci U S A. 1998;95(4):1746-1751.

372. Plotkin SA. Immunologic correlates of protection induced by vaccination. Pediatr Infect Dis J. 2001;20(1):63-75.

373. Plotkin SA. Complex correlates of protection after vaccination. Clin Infect Dis. 2013;56(10):1458-1465.

374. Plotkin SA. Updates on immunologic correlates of vaccine-induced protection. Vaccine. 2020;38(9):2250-2257.

375. Plotkin SA, Gilbert PB. Nomenclature for immune correlates of protection after vaccination. Clin Infect Dis. 2012;54(11):1615-1617.

376. Polack FP, Auwaerter PG, Lee SH, et al. Production of atypical measles in rhesus macaques: evidence for disease mediated by immune complex formation and eosinophils in the presence of fusion-inhibiting antibody. Nat Med. 1999;5(6):629-634.

377. Polack FP, Hoffman SJ, Crujeiras G, Griffin DE. A role for nonprotective complement-fixing antibodies with low avidity for measles virus in atypical measles. Nat Med. 2003;9(9):1209-1213.

378. Polack FP, Thomas SJ, Kitchin N, et al. Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. N Engl J Med. 2020;383(27):2603-2615.

379. Prince GA, Horswood RL, Chanock RM. Quantitative aspects of passive immunity to respiratory syncytial virus infection in infant cotton rats. J Virol. 1985;55(3):517-520.

380. Publicover J, Ramsburg E, Rose JK. Characterization of nonpathogenic, live, viral vaccine vectors inducing potent cellular immune responses. J Virol. 2004;78(17):9317-9324.

381. Pushko P, Parker M, Ludwig GV, Davis NL, Johnston RE, Smith JF. Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo. Virology. 1997;239(2):389-401.

382. Quadrivalent vaccine against human papillomavirus to prevent high-grade cervical lesions. N Engl J Med. 2007;356(19):1915-1927.

383. Ramon G. Sur l'augmentation anormale de l'antitoxine chez les chevaux producteurs de serum antidiptherique. Bull Soc Centr Med Vet. 1925;101:227.

384. Ranasinghe C, Ramshaw IA. Genetic heterologous prime-boost vaccination strategies for improved systemic and mucosal immunity. Expert Rev Vaccines. 2009;8(9):1171-1181.

385. Ray P, Black S, Shinefield H, et al. Risk of chronic arthropathy among women after rubella vaccination. Vaccine Safety Datalink Team [see comments]. JAMA. 1997;278(7):551-556.

386. Redfield RR, Wright DC, James WD, Jones TS, Brown C, Burke DS. Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. N Engl J Med. 1987;316:673-676.

387. Reduction of respiratory syncytial virus hospitalization among premature infants and infants with bronchopulmonary dysplasia using respiratory syncytial virus immune globulin prophylaxis. The PREVENT Study Group. Pediatrics. 1997;99(1):93-99.

388. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, et al. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N Engl J Med. 2009;361(23):2209-2220.

389. Rezapkin GV, Fan L, Asher DM, Fibi MR, Dragunsky EM, Chumakov KM. Mutations in Sabin 2 Strain of poliovirus and stability of attenuation phenotype. Virology. 1999;258(1):152-160.

390. Richman DD, Wrin T, Little SJ, Petropoulos CJ. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc Natl Acad Sci U S A. 2003;100(7):4144-4149.

391. Roberts SR, Lichtenstein D, Ball LA, Wertz GW. The membrane-associated and secreted forms of the respiratory syncytial virus attachment glycoprotein G are synthesized from alternative initiation codons. J Virol. 1994;68(7):4538-4546.

392. Robertson JS, Cook P, Nicolson C, Newman R, Wood JM. Mixed populations in influenza virus vaccine strains. Vaccine. 1994;12(14):1317-1322.

393. Roivainen M, Piirainen L, Hovi T. Persistence and class-specificity of neutralizing antibody response induced by trypsin-cleaved type 3 poliovirus in mice. Vaccine. 1993;11(7):713-717.

394. Rojas M, Donahue JP, Tan Z, Lin YZ. Genetic engineering of proteins with cell membrane permeability. Nat Biotechnol. 1998;16(4):370-375.

395. Roldao A, Mellado MC, Castilho LR, Carrondo MJ, Alves PM. Virus-like particles in vaccine development. Expert Rev Vaccines. 2010;9(10):1149-1176.

396. Romanowski B. Long term protection against cervical infection with the human papillomavirus: review of currently available vaccines. Hum Vaccin. 2011;7(2):161-169.

397. Rosario M, Hopkins R, Fulkerson J, et al. Novel recombinant *Mycobacterium bovis* BCG, ovine atadenovirus, and modified vaccinia virus Ankara vaccines combine to induce robust human immunodeficiency virus-specific CD4 and CD8 T-cell responses in rhesus macaques. J Virol. 2010;84(12):5898-5908.

398. Rose NF, Marx PA, Luckay A, et al. An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. Cell. 2001;106(5):539-549.

399. Rothenfusser S, Tuma E, Endres S, Hartmann G. Plasmacytoid dendritic cells: the key to CpG. Hum Immunol. 2002;63(12):1111-1119.

400. Rothman AL, Ennis FA. Immunopathogenesis of Dengue hemorrhagic fever. Virology. 1999;257(1):1-6.

401. Roy MJ, Wu MS, Barr LJ, et al. Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine. 2000;19(7-8):764-778.

402. Ruggeri FM, Johansen K, Basile G, Kraehenbuhl JP, Svensson L. Antirotavirus immunoglobulin A neutralizes virus in vitro after transcytosis through epithelial cells and protects infant mice from diarrhea. J Virol. 1998;72(4):2708-2714.

403. Russell SM, Liew FY. T cells primed by influenza virion internal components can cooperate in the antibody response to haemagglutinin. Nature. 1979;280(5718):147-148.

404. Sabin A. Properties and behavior of orally administered attenuated poliovirus vaccine. JAMA. 1957;164:1216-1223.

405. Sabin A, Boulger L. History of Sabin attenuated poliovirus oral live vaccine strains. J Biol Stand. 1973;1:115-118.

406. Sabin AB. Oral poliovirus vaccine: history of its development and use and current challenge to eliminate poliomyelitis from the world. J Infect Dis. 1985;151(3):420-436.

407. Sadarangani M, Kollmann T, Bjornson G, et al. The Fifth International Neonatal and Maternal Immunization Symposium (INMIS 2019): Securing Protection for the Next Generation. mSphere. 2021;6(1):e00862-20.

408. Sakamoto S, Ide T, Tokiyoshi S, et al. Studies on the structures and antigenic properties of rabies virus glycoprotein analogues produced in yeast cells. Vaccine. 1999;17(3):205-218.

409. Sakurai H, Williamson RA, Crowe JE, et al. Human antibody responses to mature and immature forms of viral envelope in respiratory

syncytial virus infection: significance for subunit vaccines. J Virol. 1999;73(4):2956-2962.

410. Salamanca de la Cueva I, Pahud B, Huang LM, et al. Immunogenicity and safety of porcine circovirus-free human rotavirus vaccine in healthy infants: a phase III, randomized trial. J Infect Dis. 2020;225(12):2106-2115.

411. Salimi N, Edwards L, Foos G, et al. A behind-the-scenes tour of the IEDB curation process: an optimized process empirically integrating automation and human curation efforts. Immunology. 2020;161(2):139-147.

412. Sambandamurthy VK, Jacobs WR Jr. Live attenuated mutants of *Mycobacterium tuberculosis* as candidate vaccines against tuberculosis. Microbes Infect. 2005;7(5-6):955-961.

413. Sanders MT, Brown LE, Deliyannis G, Pearse MJ. ISCOM-based vaccines: the second decade. Immunol Cell Biol. 2005;83(2):119-128.

414. Saphire EO, Schendel SL, Fusco ML, et al. Systematic analysis of monoclonal antibodies against Ebola virus GP defines features that contribute to protection. Cell. 2018;174(4):938-952 e13.

415. Saraiya M, Unger ER, Thompson TD, et al. US assessment of HPV types in cancers: implications for current and 9-valent HPV vaccines. J Natl Cancer Inst. 2015;107(6):djv086.

416. Sardesai NY, Weiner DB. Electroporation delivery of DNA vaccines: prospects for success. Curr Opin Immunol. 2011;23(3):421-429.

417. Schelonka RL, Raaphorst FM, Infante D, Kraig E, Teale JM, Infante AJ. T cell receptor repertoire diversity and clonal expansion in human neonates. Pediatr Res. 1998;43(3):396-402.

418. Scherle PA, Palladino G, Gerhard W. Mice can recover from pulmonary influenza virus infection in the absence of class I-restricted cytotoxic T cells. J Immunol. 1992;148(1):212-217.

419. Schlesinger JJ, Foltzer M, Chapman S. The Fc portion of antibody to yellow fever virus NS1 is a determinant of protection against YF encephalitis in mice. Virology. 1993;192(1):132-141.

420. Schmitz JE, Kuroda MJ, Santra S, et al. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science. 1999;283(5403):857-860.

421. Schnepp BC, Clark KR, Klemanski DL, Pacak CA, Johnson PR. Genetic fate of recombinant adeno-associated virus vector genomes in muscle. J Virol. 2003;77(6):3495-3504.

422. Schoeder CT, Schmitz S, Adolf-Bryfogle J, et al. Modeling immunity with Rosetta: methods for antibody and antigen design. Biochemistry. 2021;60(11):825-846.

423. Seaman MS, Xu L, Beaudry K, et al. Multiclade human immunodeficiency virus type 1 envelope immunogens elicit broad cellular and humoral immunity in rhesus monkeys. J Virol. 2005;79(5):2956-2963.

424. Seder RA, Ahmed R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat Immunol. 2003;4(9):835-842.

425. Selin LK, Lin MY, Kraemer KA, et al. Attrition of T cell memory: selective loss of LCMV epitope-specific memory CD8 T cells following infections with heterologous viruses. Immunity. 1999;11(6):733-742.

426. Selin LK, Welsh RM. Plasticity of T cell memory responses to viruses. Immunity. 2004;20(1):5-16.

427. Setliff I, Shiakolas AR, Pilewski KA, et al. High-throughput mapping of B cell receptor sequences to antigen specificity. Cell. 2019;179(7):1636-1646 e15.

428. Shiver JW, Fu TM, Chen L, et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature. 2002;415(6869): 331-335.

429. Sibbet G, Romero-Graillet C, Meneguzzi G, Campo MS. alpha6 integrin is not the obligatory cell receptor for bovine papillomavirus type 4. J Gen Virol. 2000;81(Pt 2):327-334.

430. Siegrist CA, Aspinall R. B-cell responses to vaccination at the extremes of age. Nat Rev Immunol. 2009;9(3):185-194.

431. Simoes EAF, Forleo-Neto E, Geba GP, et al. Suptavumab for the prevention of medically attended respiratory syncytial virus infection in preterm infants. Clin Infect Dis. 2021;73(11):e4400-e4408.

432. Simonsen L, Viboud C, Elixhauser A, Taylor RJ, Kapikian AZ. More on RotaShield and intussusception: the role of age at the time of vaccination. J Infect Dis. 2005;192(Suppl 1):S36-S43.

433. Singh RA, Wu L, Barry MA. Generation of genome-wide CD8 T cell responses in HLA-A*0201 transgenic mice by an HIV-1 ubiquitin expression library immunization vaccine. J Immunol. 2002;168(1):379-391.

434. Song JM, Van Rooijen N, Bozja J, Compans RW, Kang SM. Vaccination inducing broad and improved cross protection against multiple subtypes of influenza A virus. Proc Natl Acad Sci U S A. 2011;108(2):757-761.

435. Spear GT, Takefman DM, Sullivan BL, Landay AL, Jennings MB, Carlson JR. Anti-cellular antibodies in sera from vaccinated macaques can induce complement-mediated virolysis of human immunodeficiency virus and simian immunodeficiency virus. Virology. 1993;195(2):475-480.

436. Spice AJ, Aw R, Bracewell DG, Polizzi KM. Synthesis and assembly of hepatitis B virus-like particles in a Pichia pastoris cell-free system. Front Bioeng Biotechnol. 2020;8:72.

437. Spriggs MK. One step ahead of the game: viral immunomodulatory molecules. Annu Rev Immunol. 1996;14:101-130.

438. Stanberry LR, Spruance SL, Cunningham AL, et al. Glycoprotein-D-adjuvant vaccine to prevent genital herpes. N Engl J Med. 2002;347(21):1652-1661.

439. Stickl H, Hochstein-Mintzel V, Mayr A, Huber HC, Schafer H, Holzner A. MVA vaccination against smallpox: clinical tests with an attenuated live vaccinia virus strain (MVA) (author's transl). Dtsch Med Wochenschr. 1974;99(47):2386-2392.

440. Strasser R, Castilho A, Stadlmann J, et al. Improved virus neutralization by plant-produced anti-HIV antibodies with a homogeneous beta1,4-galactosylated N-glycan profile. J Biol Chem. 2009;284(31):20479-20485.

441. Strickler HD, Rosenberg PS, Devesa SS, Hertel J, Fraumeni JF Jr, Goedert JJ. Contamination of poliovirus vaccines with simian virus 40 (1955-1963) and subsequent cancer rates. JAMA. 1998;279(4):292-295.

442. Sullivan NJ, Martin JE, Graham BS, Nabel GJ. Correlates of protective immunity for Ebola vaccines: implications for regulatory approval by the animal rule. Nat Rev Microbiol. 2009;7(5):393-400.

443. Sumida SM, Truitt DM, Lemckert AA, et al. Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. J Immunol. 2005;174(11):7179-7185.

444. Tacket CO. Plant-derived vaccines against diarrheal diseases. Vaccine. 2005;23(15):1866-1869.

445. Takafuji ET, Gaydos JC, Allen RG, Top FH Jr. Simultaneous administration of live, enteric-coated adenovirus types 4, 7 and 21 vaccines: safety and immunogenicity. J Infect Dis. 1979;140(1):48-53.

446. Tan M, Huang P, Xia M, et al. Norovirus P particle, a novel platform for vaccine development and antibody production. J Virol. 2011;85(2):753-764.

447. Tan M, Jiang X. The p domain of norovirus capsid protein forms a subviral particle that binds to histo-blood group antigen receptors. J Virol. 2005;79(22):14017-14030.

448. Tang DC, DeVit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. Nature. 1992;356(6365):152-154.

449. Tao T, Skiadopoulos MH, Durbin AP, Davoodi F, Collins PL, Murphy BR. A live attenuated chimeric recombinant parainfluenza virus (PIV) encoding the internal proteins of PIV type 3 and the surface glycoproteins of PIV type 1 induces complete resistance to PIV1 challenge and partial resistance to PIV3 challenge. Vaccine. 1999;17:1100-1108.

450. Tariq H, Batool S, Asif S, Ali M, Abbasi BH. Virus-like particles: revolutionary platforms for developing vaccines against emerging infectious diseases. Front Microbiol. 2021;12:790121.

451. Taylor PM, Esquivel F, Askonas BA. Murine CD4+ T cell clones vary in function in vitro and in influenza infection in vivo. Int Immunol. 1990;2(4):323-328.

452. Theiler M, Smith H. The effect of prolonged cultivation in vitro upon the pathogenicity of yellow fever virus. J Exp Med. 1939;65:767-787.

453. Tonkin DR, Jorquera P, Todd T, Beard CW, Johnston RE, Barro M. Alphavirus replicon-based enhancement of mucosal and systemic immunity is linked to the innate response generated by primary immunization. Vaccine. 2010;28(18):3238-3246.

454. Topham DJ, Doherty PC. Clearance of an influenza A virus by CD4+ T cells is inefficient in the absence of B cells. J Virol. 1998;72(1):882-885.

455. Topham DJ, Tripp RA, Doherty PC. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. J Immunol. 1997;159(11):5197-5200.

456. Toran JL, Sanchez-Pulido L, Kremer L, del Real G, Valencia A, Martinez AC. Improvement in affinity and HIV-1 neutralization by somatic mutation in the heavy chain first complementarity-determining region of antibodies triggered by HIV-1 infection. Eur J Immunol. 2001;31(1):128-137.

457. Trapp S, von Einem J, Hofmann H, et al. Potential of equine herpesvirus 1 as a vector for immunization. J Virol. 2005;79(9):5445-5454.

458. Treanor JJ, Betts RF. Evaluation of live, cold-adapted influenza A and B virus vaccines in elderly and high-risk subjects. Vaccine. 1998;16(18):1756-1760.

459. Trill JJ, Shatzman AR, Ganguly S. Production of monoclonal antibodies in COS and CHO cells. Curr Opin Biotechnol. 1995;6(5):553-560.

460. Tripathy S, Dassarma B, Bhattacharya M, Matsabisa MG. Plant-based vaccine research development against viral diseases with emphasis on Ebola virus disease: a review study. Curr Opin Pharmacol. 2021;60:261-267.

461. U.S. Food and Drug Administration. Accessed July 13, 2022. https://www.fda.gov/vaccines-blood-biologics/vaccines/approved-vaccine-products

462. U.S. Food and Drug Administration. Accessed July 13, 2022. https://www.fda.gov/vaccines-blood-biologics/biologics-guidances/vaccine-and-related-biological-product-guidances

463. van Drunen Littel-van den Hurk S, Parker MD, Massie B, et al. Protection of cattle from BHV-1 infection by immunization with recombinant glycoprotein gIV. Vaccine. 1993;11(1):25-35.

464. Van Kampen KR, Shi Z, Gao P, et al. Safety and immunogenicity of adenovirus-vectored nasal and epicutaneous influenza vaccines in humans. Vaccine. 2005;23(8): 1029-1036.

465. Vasan S, Hurley A, Schlesinger SJ, et al. In vivo electroporation enhances the immunogenicity of an HIV-1 DNA vaccine candidate in healthy volunteers. PLoS One. 2011;6(5):e19252.

466. Vazquez M, LaRussa PS, Gershon AA, et al. Effectiveness over time of varicella vaccine. JAMA. 2004;291(7):851-855.

467. Vennema H, de Groot RJ, Harbour DA, et al. Early death after feline infectious peritonitis virus challenge due to recombinant vaccinia virus immunization. J Virol. 1990;64(3):1407-1409.

468. Victoria JG, Wang C, Jones MS, et al. Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. J Virol. 2010;84(12):6033-6040.

469. Villa LL, Costa RL, Petta CA, et al. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. Lancet Oncol. 2005;6(5):271-278.

470. Walker LM, Phogat SK, Chan-Hui PY, et al. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science. 2009;326(5950):285-289.

471. Walls AC, Fiala B, Schafer A, et al. Elicitation of potent neutralizing antibody responses by designed protein nanoparticle vaccines for SARS-CoV-2. Cell. 2020;183(5):1367-1382 e17.

472. Wan H, Gao J, Yang H, et al. The neuraminidase of A(H3N2) influenza viruses circulating since 2016 is antigenically distinct from the A/Hong Kong/4801/2014 vaccine strain. Nat Microbiol. 2019;4(12):2216-2225.

473. Wang BZ, Xu R, Quan FS, Kang SM, Wang L, Compans RW. Intranasal immunization with influenza VLPs incorporating membrane-anchored flagellin induces strong heterosubtypic protection. PLoS One. 2010;5(11):e13972.

474. Wang C, Ge Q, Ting D, et al. Molecularly engineered poly(ortho ester) microspheres for enhanced delivery of DNA vaccines. Nat Mater. 2004;3(3):190-196.

475. Wang R, Doolan DL, Le TP, et al. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. Science. 1998;282(5388):476-480.

476. Wang TT, Maamary J, Tan GS, et al. Anti-HA Glycoforms drive B cell affinity selection and determine influenza vaccine efficacy. Cell. 2015;162(1):160-169.

477. Wang TT, Tan GS, Hai R, et al. Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. Proc Natl Acad Sci U S A. 2010;107(44):18979-18984.

478. Watanabe A, McCarthy KR, Kuraoka M, et al. Antibodies to a conserved influenza head interface epitope protect by an IgG subtype-dependent mechanism. Cell. 2019;177(5):1124-1135 e16.

479. Waters TD, Anderson PS Jr, Beebe GW, Miller RW. Yellow fever vaccination, avian leukosis virus, and cancer risk in man. Science. 1972;177(43):76-77.

480. Weber J, Cheinsong-Popov R, Callow D, et al. Immunogenicity of the yeast recombinant p17/p24:Ty virus-like particles (p24-VLP) in healthy volunteers. Vaccine. 1995;13(9):831-834.

481. Wec AZ, Nyakatura EK, Herbert AS, et al. A “Trojan horse” bispecific-antibody strategy for broad protection against ebolaviruses. Science. 2016;354(6310):350-354.

482. Weitkamp JH, Lafleur BJ, Greenberg HB, Crowe JE Jr. Natural evolution of a human virus-specific antibody gene repertoire by somatic hypermutation requires both hotspot-directed and randomly-directed processes. Hum Immunol. 2005;66(6):666-676.

483. Werner GT, Jentzsch U, Metzger E, Simon J. Studies on Poxvirus infections in irradiated animals. Arch Virol. 1980;64:247-256.

484. Wessel AW, Doyle MP, Engdahl TB, Rodriguez J, Crowe JE Jr, Diamond MS. Human monoclonal antibodies against NS1 protein protect against lethal West Nile virus infection. MBio. 2021;12(5):e0244021.

485. Wille-Reece U, Wu CY, Flynn BJ, Kedl RM, Seder RA. Immunization with HIV-1 Gag protein conjugated to a TLR7/8 agonist results in the generation of HIV-1 Gag-specific Th1 and CD8+ T cell responses. J Immunol. 2005;174(12):7676-7683.

486. Williamson C, Morris L, Maughan MF, et al. Characterization and selection of HIV-1 subtype C isolates for use in vaccine development. AIDS Res Hum Retroviruses. 2003;19(2):133-144.

487. Wilson AD, Lovgren-Bengtsson K, Villacres-Ericsson M, Morein B, Morgan AJ. The major Epstein-Barr virus (EBV) envelope glycoprotein gp340 when incorporated into Iscoms primes cytotoxic T-cell responses directed against EBV lymphoblastoid cell lines. Vaccine. 1999;17(9-10):1282-1290.

488. Wolf M, Garcea RL, Grigorieff N, Harrison SC. Subunit interactions in bovine papillomavirus. Proc Natl Acad Sci U S A. 2010;107(14):6298-6303.

489. Wolff JA, Malone RW, Williams P, et al. Direct gene transfer into mouse muscle in vivo. Science. 1990;247(4949 Pt 1):1465-1468.

490. Wood RC, MacDonald KL, White KE, Hedberg CW, Hanson M, Osterholm MT. Risk factors for lack of detectable antibody following hepatitis B vaccination of Minnesota health care workers. JAMA. 1993;270(24):2935-2939.

491. Worobey M, Santiago ML, Keele BF, et al. Origin of AIDS: contaminated polio vaccine theory refuted. Nature. 2004;428(6985):820.

492. Wrammert J, Smith K, Miller J, et al. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. Nature. 2008;453(7195):667-671.

493. Wright PF, Karron RA, Belshe RB, et al. Evaluation of a live, cold-passaged, temperature-sensitive, respiratory syncytial virus vaccine candidate in infancy. J Infect Dis. 2000;182(5):1331-1342.

494. Wu H, Pfarr DS, Tang Y, et al. Ultra-potent antibodies against respiratory syncytial virus: effects of binding kinetics and binding valence on viral neutralization. J Mol Biol. 2005;350(1):126-144.

495. Wu X, Yang ZY, Li Y, et al. Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. Science. 2010;329(5993):856-861.

496. Xin KQ, Jounai N, Someya K, et al. Prime-boost vaccination with plasmid DNA and a chimeric adenovirus type 5 vector with type 35 fiber induces protective immunity against HIV. Gene Ther. 2005;12(24):1769-1777.

497. Xing L, Wang JC, Li TC, et al. Spatial configuration of hepatitis E virus antigenic domain. J Virol. 2011;85(2):1117-1124.

498. Yan H, Lamm ME, Bjorling E, Huang YT. Multiple functions of immunoglobulin A in mucosal defense against viruses: an in vitro measles virus model. J Virol. 2002;76(21):10972-10979.

499. Yang OO, Kalams SA, Trocha A, et al. Suppression of human immunodeficiency virus type 1 replication by CD8+ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. J Virol. 1997;71(4):3120-3128.

500. Yang Y, Shi W, Abiona OM, et al. Newcastle disease virus-like particles displaying prefusion-stabilized SARS-CoV-2 spikes elicit potent neutralizing responses. Vaccines (Basel). 2021;9(2):73.

501. Yassine HM, Boyington JC, McTamney PM, et al. Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. Nat Med. 2015;21(9):1065-1070.

502. Yokomori K, Baker SC, Stohlman SA, Lai MM. Hemagglutinin-esterase-specific monoclonal antibodies alter the neuropathogenicity of mouse hepatitis virus. J Virol. 1992;66(5):2865-2874.

503. Yu X, Tsibane T, McGraw PA, et al. Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. Nature. 2008;455(7212):532-536.

504. Yu Z, Karem KL, Kanangat S, Manickan E, Rouse BT. Protection by minigenes: a novel approach of DNA vaccines. Vaccine. 1998;16(17):1660-1667.

505. Yuan L, Ward LA, Rosen BI, To TL, Saif LJ. Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease. J Virol. 1996;70(5):3075-3083.

506. Zagury D. Anti-HIV cellular immunotherapy in AIDS. Lancet. 1991;338(8768):694-695.

507. Zaia JA, Levin MJ, Preblud SR, et al. Evaluation of varicella-zoster immune globulin: protection of immunosuppressed children after household exposure to varicella. J Infect Dis. 1983;147(4):737-743.

508. Zhang X, Brunner T, Carter L, et al. Unequal death in T helper cell (Th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. J Exp Med. 1997;185(10):1837-1849.

509. Zhou J, Sun XY, Stenzel DJ, Frazer IH. Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. Virology. 1991;185(1):251-257.

510. Zhou T, Georgiev I, Wu X, et al. Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. Science. 2010;329(5993):811-817.

511. Zhu FC, Zhang J, Zhang XF, et al. Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: a large-scale, randomised, double-blind placebo-controlled, phase 3 trial. Lancet. 2010;376(9744):895-902.

512. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations. Annu Rev Immunol. 2010;28:445-489.

513. Zost SJ, Gilchuk P, Chen RE, et al. Rapid isolation and profiling of a diverse panel of human monoclonal antibodies targeting the SARS-CoV-2 spike protein. Nat Med. 2020;26(9):1422-1427.

514. Zost SJ, Parkhouse K, Gumina ME, et al. Contemporary H3N2 influenza viruses have a glycosylation site that alters binding of antibodies elicited by egg-adapted vaccine strains. Proc Natl Acad Sci U S A. 2017;114(47):12578-12583.

515. Zost SJ, Wu NC, Hensley SE, Wilson IA. Immunodominance and antigenic variation of influenza virus hemagglutinin: implications for design of universal vaccine immunogens. J Infect Dis. 2019;219(Suppl_1):S38-S45.

CHAPTER 16

Diagnostic Virology

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Introduction

Diagnostic virology continues to evolve rapidly. Viral testing is essential for the care of hospitalized patients with acute respiratory infections; transplant recipients and other immunocompromised patients; patients infected with human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV); and infants with possible congenital infection, and many others. Diagnostic virology also stands as the first line of defense against the emergence of new viruses in the human population. Multiple test methods can be useful, but molecular tests have emerged as the dominant diagnostic technology with rapid point-of-care antigen tests serving as an additional tool. A variety of highly automated commercial molecular assays have been approved or cleared as *in vitro* diagnostic tests by the Food and Drug Administration (FDA). This is an important development because it makes viral diagnostic testing available to more laboratories, and it improves the standardization of diagnostic testing. Furthermore, the emergence of rapid, multiplex syndromic panels has reshaped diagnostic virology. The scope of diagnostic virology has broadened, especially in the wake of the COVID-19 pandemic. General categories of viral diagnostic testing and the viruses included in those categories are shown in Table 16.1.

TABLE 16.1 Categories of testing performed in diagnostic virology

Category of Testing	Specific Viruses	Methodology
Respiratory viruses	Influenza A and B, RSV, PIV 1-4, hMPV, rhinoviruses, enteroviruses, coronaviruses, adenoviruses	Rapid antigen tests (SARS-CoV-2, influenza A and B, RSV), fluorescent antibody staining, culture, multiplex NAAT
Gastrointestinal viruses	Rotavirus, norovirus, adenovirus, astrovirus	Rapid antigen tests (rotavirus, norovirus, adenovirus), NAAT
Mucocutaneous viruses	HSV, VZV, HPV	Fluorescent antibody staining (HSV and VZV), culture (HSV and VZV), NAAT
Central nervous system viruses	HSV, VZV, CMV, EBV, HHV-6, JCV, enteroviruses, parechoviruses, West Nile virus, other arboviruses	NAAT, serology (West Nile and other arboviruses)
Opportunistic agents	CMV, EBV, BKV, HHV-6, adenoviruses	NAAT, serology
Mononucleosis syndrome in nonimmunocompromised individuals	EBV, CMV, HIV	Serology, NAAT
HIV, HCV, HBV viral loads	HIV, HCV, HBV	NAAT
Viral genotyping	HCV, HBV, HPV, HIV, CMV	Nucleotide sequencing, reverse hybridization, NAAT (cleavase reaction for HPV)
HIV, HCV, HBV diagnosis	HIV, HCV, HBV	Serology, NAAT
Systemic infections of childhood	Parvovirus B19, measles virus, rubella virus, mumps virus	NAAT, serology
Tropical and emerging infections	Dengue and other flaviviruses; chikungunya and other alphaviruses; hemorrhagic fever viruses including arenaviruses, bunyaviruses, and filoviruses; Hendra and Nipah viruses	Serology, culture, NAAT (hemorrhagic fever testing is done in BSL-4 laboratories)
Unknown virus	Any	Culture, metagenomic next-generation sequencing

BKV, BK virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HHV-6, human herpesvirus 6; HIV, human immunodeficiency virus; hMPV, human metapneumovirus; HPV, human papillomavirus; HSV, herpes simplex virus; JCV, JC virus; NAAT, nucleic acid amplification testing; PIV, parainfluenza virus; RSV, respiratory syncytial virus; VZV, varicella-zoster virus.

History of Diagnostic Virology

Modern diagnostic virology dates to the first growth of human viruses in tissue culture reported by Weller and Enders in 1948.[84] This and other landmarks in the history of diagnostic virology are shown in Table 16.2.

TABLE 16.2 Landmarks in the history of diagnostic virology

Year	Landmark
1892	Intranuclear and intracytoplasmic inclusions noted at the base of smallpox lesions
1898	Discovery by Loeffler and Frosch that foot-and-mouth disease of cattle is caused by a filterable agent, referred to as a virus
1929	Complement fixation method described for detection of antibodies to smallpox, vaccinia, and varicella-zoster viruses[3]
1948	First growth of pathogenic human viruses in tissue culture[84]
1956	Detection of influenza virus in respiratory secretions using fluorescent antibody staining[45]
1975	Development of monoclonal antibodies as diagnostic reagents[42]
1985	Discovery of PCR[69]
1992	Development of real-time PCR[34]
2002	Beginning of systematic approaches to virus discovery[27,31]
2007	Description of multiplex PCR for respiratory viruses[47]
2020-2021	Widespread diagnostic testing for SARS-CoV-2 for public health purposes, including availability of at-home testing

PCR, polymerase chain reaction.

Specimens for Viral Diagnosis

The ability to make a specific viral diagnosis relies on the quality of the specimen that is received in the laboratory. Important variables include the timing of the specimen in relation to the patient's illness, the type of specimen, the quality and amount of specimen material obtained, and the time and conditions of transport to the laboratory. Optimizing the variables listed requires knowledge on the part of the care provider and close attention to logistical considerations.

For the diagnosis of acute viral infections, the best specimens are usually obtained from the site of disease. For example, in the patient with suspected viral meningitis, cerebrospinal fluid (CSF) is the best specimen. In infections involving skin or mucosal surfaces, specimens obtained from those surfaces are usually the only ones required. Viral quantities are often highest early in the course of an acute illness, so that specimens obtained within the first few days after onset are most likely to be positive.

Viral culture requires more attention to conditions of transport than specimens submitted for detection of viral antigens or nucleic acids, because the viability of the virus must be preserved. A number of clinically important viruses are labile and will not survive prolonged transport. Consultation with the laboratory performing testing is generally advised.

For serologic diagnosis, an acute phase serum specimen should be obtained within the first few days of illness and a convalescent phase specimen 2-4 weeks later. If a virus-specific immunoglobulin M (IgM) assay is available, the acute phase specimen may be sufficient by itself. For certain viral infections, serologic tests can also be performed on saliva, CSF, or urine.

Methods Used in Diagnostic Virology

Viral Culture

For almost 50 years, viral culture was the signature method of diagnostic virology laboratories, and it was the method that led to the establishment of virology laboratories as distinct entities apart from other areas of the clinical laboratory. With the advent of immunological and molecular methods, which are more rapid than viral culture and potentially detect a broader range of viruses, cell culture has been eliminated by some diagnostic virology laboratories. For this reason, the treatment of cell culture in this chapter will be brief, and the interested reader is referred to other sources such as ASM's *Manual of Clinical Microbiology* or Lennette's *Laboratory Diagnosis of Viral Infection*.

By their very nature, viruses require living systems for propagation. Initially, inoculation of animals such as suckling mice or embryonated eggs was used to grow viruses. Such methods were subsequently supplanted by cell culture in the diagnostic laboratory. To grow viruses in cell culture, a clinical sample is prepared and then inoculated onto one or more cell lines (e-Table 16.1). Typically, several cell culture types are inoculated, because no one type supports the growth of all clinically relevant viruses. Cell culture types that are commonly used are shown in Table 16.3. After inoculation, viral growth is detected in one of several ways. Traditionally, the principal method was the appearance of cytopathic effect, which refers to morphologic changes observable by microscopy that occur in virally infected cells (Fig. 16.1). Confirmation of

virus growth, suspected based on one of the indicators described previously, can be achieved by immunofluorescence or by molecular analysis. In puzzling cases, electron microscopy can be used to look for viral particles that would confirm viral infection and might provide taxonomic information based on morphologic characteristics. Other methods that have been used to enhance viral cultures are use of hemadsorption to detect viral growth, centrifugation (including shell-vial cultures, e-Fig. 16.1), mixed cell type cultures, and use of cells that have been genetically engineered to enhance viral growth and/or detection in culture.

TABLE 16.3 Cell culture types used to detect medically important viruses

Cell Culture Type	Cell Type	Viruses Detected
Primary	Monkey kidney	Influenza viruses, parainfluenza viruses, enteroviruses
	Rabbit kidney	Herpes simplex virus
	Human embryonic kidney	Adenovirus, enteroviruses
Diploid	Fibroblasts (eg, MRC-5, WI-38)	CMV, VZV, HSV, rhinovirus, enteroviruses (some), adenovirus, RSV
Continuous	HEp-2	RSV, adenovirus, HSV, parainfluenza viruses (some), enteroviruses (some)
	A549	HSV, adenovirus, enteroviruses
	MDCK	Influenza viruses
	LLC-MK2	Parainfluenza viruses, hMPV
	Rhabdosarcoma (RD)	Echoviruses
	Buffalo green monkey	Coxsackieviruses

CMV, cytomegalovirus; hMPV, human metapneumovirus; HSV, herpes simplex virus; RSV, respiratory syncytial virus; VZV, varicella-zoster virus.

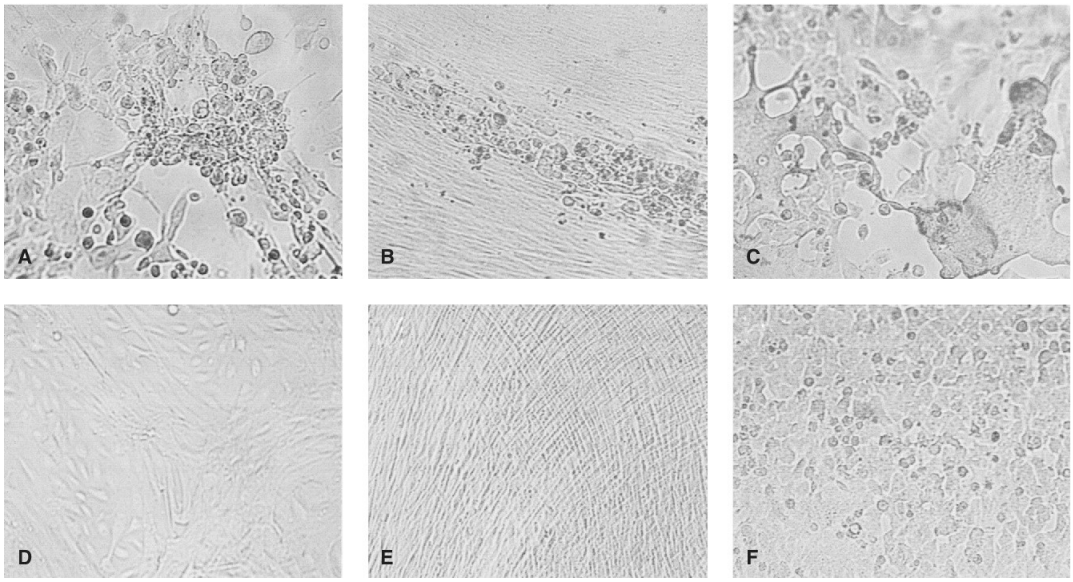
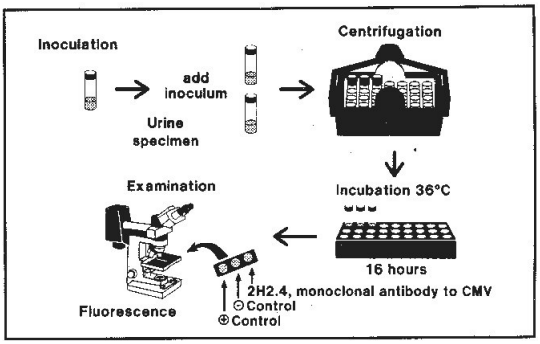


FIGURE 16.1 **Cytopathic effect caused by viruses growing in cell culture**. Herpes simplex virus growing in primary rabbit kidney cells **(A)**; uninfected primary rabbit kidney cells **(D)**. Cytomegalovirus growing in human embryonic lung fibroblast cells **(B)**; uninfected human embryonic lung fibroblast cells **(E)**. Respiratory syncytial virus growing in HEp-2 cells **(C)**; uninfected HEp-2 cells **(F)**.



e-FIGURE 16.1 **Shell-vial assay for cytomegalovirus.** (From Shuster EA, Beneke JS, Tegtmeier GE, et al. Monoclonal antibody for rapid laboratory detection of cytomegalovirus infections: characterization and diagnostic application. *Mayo Clin Proc*. 1985;60:577-585, with permission.)

e-TABLE 16.1 Instructions for obtaining and transporting specimens for viral testing[a]

Specimen	Instructions[b]
Blood	Volume requirement varies according to the specific test required. Molecular methods generally require lower blood volumes than does culture. The appropriate tube is determined by the test to be performed, the blood component on which the test is performed (whole blood, leukocytes, plasma, or serum), and the preference of the laboratory. Whole blood, leukocytes, and plasma require collection into a tube containing an anticoagulant. The most commonly used anticoagulants are ethylenediaminetetraacetic acid (EDTA) (purple top tube), heparin (green top tube), and acid-citrate-dextrose (yellow top tube). Note: Heparin is inhibitory for some polymerase chain reaction (PCR) procedures, especially those performed on plasma. Some nonculture tests performed on blood may have specific collection tube requirements that can be determined through communication with the laboratory. Most serologic procedures are performed on serum, although some tests can also be performed on plasma. Serum specimens require collection into a tube that does not contain an anticoagulant (red top tube or gold top serum separator tubes). Blood specimens do not have to be placed on ice if transport requires >1 day. Note: Prolonged transport can severely compromise the ability to recover cytomegalovirus (CMV) in culture from blood specimens.[c]
Swab	Swabs with Dacron or rayon tips are preferred. If available, swabs designed specifically for collection of viral specimens should be used. Recently, flocked swabs have been shown to be superior to standard swabs.[d,e] Swabs can also be placed in viral transport media. In this case, the shaft of the swab may have to be cut to allow the top of the transport vial to be securely closed. The swab or tube of transport media should be placed on ice if transport will require more than 1 hour.
Fluid	Place in a sterile container.
Tissue	Place in a sterile container with a small amount of viral transport media or sterile saline or phosphate-buffered saline to maintain moisture.
Stool	Obtain at least 4 g of stool. Place in a clean or sterile container.

[a]Specimens other than blood should be transported on ice if more than 1 hour is required for arrival in the laboratory. If a delay of more than 24 hours is anticipated, specimens should be frozen at −70 °C and transported on dry ice. Note: Some viruses such as respiratory syncytial virus (RSV) and varicella-zoster virus (VZV) are unlikely to retain viability with freezing. Specimens should not be frozen at −20 °C.

[b]Commercial tests may have specific recommendations for collection and transport. These should always be followed to ensure proper assay performance.[c]Roberts TC, Buller RS, Gaudreault-Keener M, et al. Effects of storage temperature and time on qualitative and quantitative detection of cytomegalovirus in blood specimens by shell vial culture and PCR. *J Clin Microbiol*. 1997;35:2224-2228.[d]Abu-Diab A, Azzeh M, Ghneim R, et al. Comparison between pernasal flocked swabs and nasopharyngeal aspirates for detection of common respiratory viruses in samples from children. *J Clin Microbiol*. 2008;46:2414-2417.[e]Munywoki PK, Hamid F, Mutunga M, et al. Improved detection of respiratory viruses in pediatric outpatients with acute respiratory illness by real-time PCR using nasopharyngeal flocked swabs. *J Clin Microbiol*. 2011;49:3365-3367.

Electron Microscopy

Historically, electron microscopy was used for the evaluation of stool specimens from patients with suspected viral gastroenteritis. Viruses such as rotavirus, astrovirus, and adenovirus could be identified based on their characteristic morphology, but this has been replaced by rapid immunoassays and PCR assays. Electron microscopy can also be used to detect and classify viral particles in fixed tissue obtained by biopsy or at autopsy. However, viral infections are rarely diagnosed using electron microscopy for the direct visualization of viral particles in specimens due to cost and complexity of maintaining an electron microscopy, the need for a skilled operator, and limited sensitivity related to the relatively high concentration of viral particles (10^5-10^6/L) that is required for visualization.

Light Microscopy

Although viral particles, with the exception of poxviruses, cannot be directly visualized by light microscopy, indirect evidence of viral infection can be detected. The most characteristic signs of viral infection are inclusion bodies (composed of masses of virions), multinucleated cells, and syncytial cells (e-Table 16.2). These findings may be present in cytologic specimens or in tissue examined after histologic staining. Cytologic examination for evidence of viral infection can be performed on smears prepared by applying a specimen directly to a microscope slide, on slides prepared by cytocentrifugation of fluids (eg, bronchoalveolar lavage fluid), and on touch preparations prepared from pieces of unfixed tissue. Microscopy for detection of the effects of viral infections in clinical specimens is generally carried out in surgical pathology rather than diagnostic virology laboratories.

e-TABLE 16.2 Viral inclusions

Virus	Nuclear	Cytoplasmic
Herpes simplex, varicella-zoster	×	
Cytomegalovirus	×	×
Adenovirus	×	
Polyomaviruses (JC and BK)	×	
Parvovirus B19	×	
Poxviruses		×
Measles	×	×
Parainfluenza		×
Rabies		×

Cytology and Histology

Cytologic examination for evidence of viral infection can be performed on smears prepared by applying a specimen directly to a microscope slide, on slides prepared by cytocentrifugation of fluids (eg, bronchoalveolar lavage fluid), and on touch preparations prepared from pieces of unfixed tissue. Viruses that can produce cytologic evidence of infection in respiratory specimens include HSV, CMV, adenoviruses, polyomaviruses, and measles virus. Cytologic examination is usually not a sensitive method for detection but provides strong evidence for tissue involvement. Examples of cytologic findings suggestive of viral infection are shown in Figure 16.2. The Tzanck smear is a method sometimes used to detect cytologic evidence of HSV or VZV infection. It is rarely used in diagnostic virology laboratories because techniques such as fluorescent antibody (FA) staining and PCR are more sensitive and specific. The Papanicolaou stain (Pap smear) is used to detect evidence of HPV infection in cells obtained from the uterine cervix. HPV infection produces characteristic changes in keratinocytes, including a condensed nucleus with a prominent perinuclear clear zone referred to as *koilocytosis*. Urine cytology may reveal intranuclear inclusions indicative of infection with either CMV or the polyomaviruses, JC and BK; however, PCR is both more sensitive and specific.

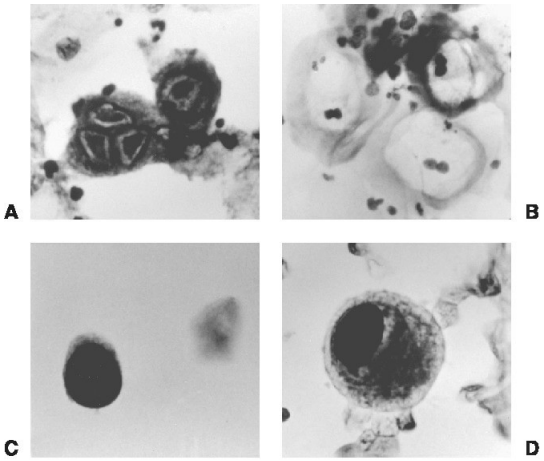


FIGURE 16.2 **Cytologic findings suggestive of viral infection**. **A**. Cervical smear showing multinucleated cells and the Cowdry type A intranuclear inclusions of herpes simplex infection. **B**. Papanicolaou smear showing binucleate squamous epithelial cells with distinct perinuclear halos. These characteristics, described as *koilocytosis*, are the cellular features associated with human papillomavirus infection. **C**. Urinary epithelial cell containing an enlarged nucleus with smudgy chromatin and a small pale *glassy* intranuclear inclusion indicative of polyomavirus infection. **D**. Cell from bronchoalveolar lavage with a large intranuclear inclusion with a perinuclear clear space (owl's eye cell) indicative of cytomegalovirus infection. (Photographs provided by Dr. Leslie Boucher, Department of Pathology, Washington University, St. Louis, Missouri.)

Antigen Detection

The detection of viral antigens directly in clinical specimens is widely used to obtain rapid evidence of viral infection. The lack of requirement for virus viability and ability to be performed at home or in a physician's office are important advantages. For most viruses, the sensitivity of antigen detection equals or exceeds that of viral culture.[64,78] Antigen detection methods can be applied when the following conditions are met: (1) viral antigen is expressed and is present in an accessible specimen, (2) an appropriate antibody (usually but not necessarily monoclonal) is available, (3) antigenic variability does not preclude recognition by immunologic reagents of different strains of the target virus, and (4) the antigen being detected is sufficiently stable that it does not degrade during transport and processing of the specimen. Methods used for viral antigen detection include FA staining, immunoperoxidase staining, and enzyme immunoassay. The latter can be subdivided into solid phase and lateral flow immunochromatographic assays.

Antigen detection can also be used to detect viral antigens in fixed tissue (immunohistochemistry [IHC]). This method can document the specific viral etiology of a nonspecific finding such as an inclusion body that could be produced by several different viruses. In some cases, IHC also enhances the sensitivity of detection of viruses in tissue compared to standard light microscopy without immunostaining. Immunoperoxidase

staining is the most common technique used for viral antigen detection in fixed tissue.

Fluorescent Antibody Staining

Fluorescent antibody staining is widely used to detect cell-associated viral antigens. In the direct format, a fluorescent label, usually fluorescein isothiocyanate (FITC), is conjugated directly to the antibody that recognizes the viral antigen. In the indirect format, the antiviral antibody is unlabeled and is detected by a second antibody that recognizes immunoglobulins from the animal species of origin of the antiviral antibody. The second antibody carries the fluorescent label. Prior to widespread molecular methods, FA staining was a common multiplexed method to detect respiratory, ocular, cutaneous, and bloodstream pathogens. Specimens are processed in the laboratory to prepare a pellet of cells, which is spotted onto one or more microscope slides. The cells are air dried, fixed in acetone, and stained with monoclonal antibodies to one or more viruses. The major limitation of FA staining is having an adequate number of cells in the specimen and low analytical sensitivity compared to molecular methods.

Enzyme Immunoassay

Enzyme immunoassay (EIA) is a versatile and widely used method that can be applied to the detection of antigens or antibodies. Because intact cells in the specimen are not required, specimen integrity is less important than for FA or IP staining. A common assay format for antigen detection is the double antibody sandwich technique, shown in Figure 16.3. In this assay, a *capture* antibody specific for the viral antigen being sought is bound to a reaction surface, for example, the wells of a plastic microtiter tray or the surface of a plastic bead. When the specimen is added, viral antigen present in the specimen binds to the capture antibody. Bound antigen is detected using a different antiviral antibody, the *detector* antibody. The detector antibody can be labeled with an enzyme or can be detected by the addition of an enzyme-labeled third antibody with specificity for immunoglobulin of the species from which the second antibody was derived. The addition of an enzyme substrate produces a color change or light emission if the enzyme is present. Thus, color change or light emission indicates that viral antigen was present in the specimen being tested. Advantages of EIA include applicability to diverse specimens and potential for automation. Laboratory instruments are now available that can perform EIA to detect either antigens or antibodies.

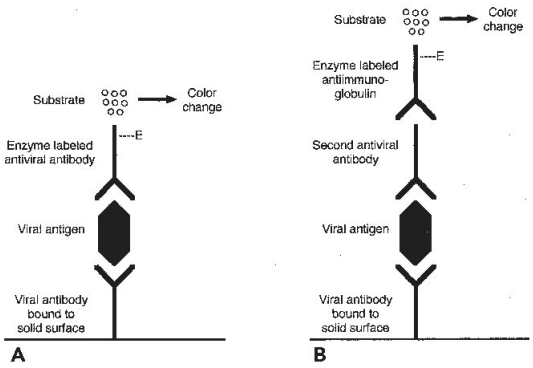


FIGURE 16.3 **Enzyme immunoassay (EIA) for antigen detection**. **A**. Direct. **B**. Indirect.

Membrane Immunoassay

The lateral flow immunochromatographic assay is a variant of EIA that was first used in home pregnancy tests. A schematic diagram of a lateral flow unit is shown in Figure 16.4. In these tests, a sample is applied directly to a membrane and is drawn across the membrane by capillary action. Antigens in the sample react first with an antibody with specificity for the antigen being detected. This antibody is conjugated to a detector label such as gold particles or fluorescein. If binding occurs, the antigen-antibody complexes sweep across the membrane until they are captured by a second antibody that is bound to the membrane. When the labeled antigen-antibody complexes are captured, a line becomes visible because of the concentration of the label into a limited physical space. Most assays also include a positive control. These assays can be configured as dipsticks or as self-contained cassettes that are amenable to highly distributed testing. Standard lateral flow immunochromatographic assays do not require an instrument and are convenient for testing single samples, with results available in 5-20 minutes. Multiple commercial versions are now available, mainly for detection of SARS-CoV-2, influenza virus, respiratory syncytial virus (RSV), and rotavirus. Many of the tests are sufficiently simple to perform that they have been assigned *waived* status under the Clinical Laboratory Improvement Amendments (CLIAs), meaning that they can be performed by individuals without specific training who can be working within or outside of a certified clinical laboratory. Sensitivity of these tests has been less than that of molecular testing. This problem was especially true for commercial influenza lateral flow tests in detecting the 2009 HIN1 pandemic. During the SARS-CoV-2 pandemic, the regulatory pathway for waived at-home antigen testing greatly eased and, as a result, tens of millions of antigen tests are now manufactured per month, opening up a new avenue for community viral diagnostics in the coming decade. These tests generally had analytical sensitivities in the tens of thousands of copies per swab, which matched that of virus culture and could help rapidly identify individuals with treatable and/or highly transmissible amounts of virus early in illness.[61] Improvements in analytical sensitivity as well as chain of custody for more regulated, diagnostic uses of these tests are expected in coming years. Addition of a detection reader may increase sensitivity of antigen tests as has been shown for RSV and influenza virus previously.[25]

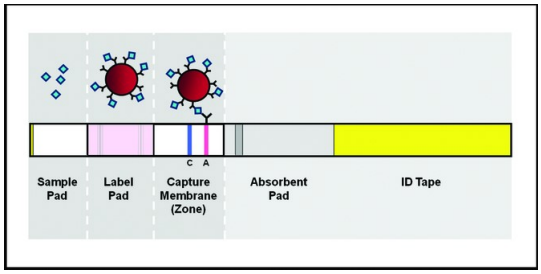


FIGURE 16.4 **Lateral flow immunochromatographic assay**. The sample is applied to the sample pad. The sample flows across the unit from left to right, drawn by the absorbent pad. Located in the label pad are one or more antibodies, each of which is conjugated to a label such as gold particles. These antibodies bind to antigen present in the sample as it moves across, forming complexes of antigen with conjugated antibody. If present, these complexes are then captured by second antibodies, located in lines in the capture membrane zone. When complexes are captured, the presence of the conjugated label causes the line to become visible. In the figure, antibodies against the target antigen are indicated by "A," and antibodies against a control antigen are indicated by "C." Presence of a colored line corresponding to A is a positive test. Presence of a line corresponding to C in the absence of a line corresponding to A indicates a negative test. Absence of both lines indicates that the test is not valid. (Figure provided by John Tamerius, Quidel Inc.)

Nucleic Acid Detection

Diagnostic virology has been revolutionized by the application of nucleic acid detection techniques, which detect specific DNA or RNA sequences, that can be applied to the detection of virtually any virus. Depending on the target sequence, the assays can be specific for a single virus species or for a group of related viruses. The latter characteristic is particularly advantageous, because it allows nucleic acid detection techniques to be applied to groups of viruses (eg, the enteroviruses/rhinoviruses) for which antigenic diversity has precluded successful application of antigen detection techniques. For example, enteroviruses, for which a rapid nonmolecular detection method had not been available, can be detected using reverse-transcription PCR assays that amplify a highly conserved sequence in the 5′ nontranslated region of the genome.[9,66] An overview of applications of nucleic acid testing in diagnostic virology is shown in e-Table 16.3.

e-TABLE 16.3 Nucleic acid detection for viral diagnosis

Target	Specimen(s)	Application(s)
HIV (DNA)	Leukocytes from infant born to HIV-infected mother	Diagnosis of perinatal infection
HIV (quantitative RNA)	Plasma	Viral load (prognosis, response to treatment)
HIV (qualitative RNA)	Plasma	Confirmation of diagnosis
HSV	CSF, ocular fluid, swabs from mucocutaneous lesions, blood	Diagnosis of encephalitis, meningitis, retinitis, mucocutaneous lesions
VZV	CSF, ocular fluid, swabs from mucocutaneous lesions, blood	Diagnosis of encephalitis, meningitis, retinitis, mucocutaneous lesions
CMV	Blood (whole blood, leukocytes, or plasma), CSF, ocular fluid, amniotic fluid	Diagnosis of systemic infection after organ transplantation, encephalitis, radiculomyelitis, retinitis, congenital infection. Quantitative assay performed on blood can be used to assess severity of infection and monitor response to therapy
EBV	CSF, blood (whole blood, leukocytes, or plasma)	Primary central nervous system lymphoma in AIDS, other EBV infection of the central nervous system, and posttransplant lymphoproliferative disorder. Quantitative assay performed on blood can be used to assess the risk of PTLD and the response to therapeutic interventions
HHV-6, -7	Blood, CSF	Diagnosis of systemic infection and of encephalitis
HHV-8 (Kaposi sarcoma virus)	Blood, pleural, or pericardial fluid	Diagnosis of body cavity lymphoma
Adenovirus	Blood, respiratory secretions, urine	Diagnosis and monitoring of disseminated infection in immunocompromised hosts, diagnosis of respiratory tract infection
Parvovirus B19	Serum, amniotic fluid	Diagnosis of acute or chronic parvovirus B19 infection, aplastic crisis, congenital infection
BK virus	Urine, plasma	Assessment of risk of transplant nephropathy in renal transplant recipients and of hemorrhagic cystitis in hematopoietic stem cell transplant recipients
Human papillomavirus	Genital secretions	Detection of viral types associated with cervical carcinoma
Enteroviruses	CSF, blood (whole blood, serum, or plasma)	Diagnosis of meningitis, encephalitis, or systemic infection
Hepatitis C	Plasma	Diagnosis of infection. Quantitative assay is used to assess response to therapy
Hepatitis B	Serum	Diagnosis of infection when serologic studies are ambiguous. Recognition of virologically active infection. Quantitative assay is used to assess response to therapy and recognize emergence of drug resistance
Rabies	Saliva, CSF	Diagnosis of rabies
Respiratory viruses	Respiratory samples	Enhanced sensitivity, including detection of noncultivable agents
Gastrointestinal viruses	Feces	Enhanced sensitivity, including detection of noncultivable agents
West Nile and other flavivirus infections	CSF, blood	Rapid diagnosis of CNS infection, especially in immunocompromised hosts, screening of blood units for presymptomatic infection
Dengue	Plasma	Diagnosis of early infection, genotyping
Arenaviruses	Serum or whole blood	Diagnosis of acute infection
Filoviruses	Serum or whole blood	Diagnosis of acute infection
Nipah and Hendra viruses	CSF, throat swabs, urine	Diagnosis of acute infection
Vaccinia and other pox virus infections	Lesion	Diagnosis of acute infection

CMV, cytomegalovirus; CNS, central nervous system; CSF, cerebrospinal fluid; EBV, Epstein-Barr virus; HHV, human herpesvirus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; PTLD, posttransplant lymphoproliferative disorder.

The earliest attempts at nucleic acid–based diagnosis involved direct hybridization of nucleic acid probes to viral nucleic acids present in clinical specimens. Direct hybridization was never widely adopted because it lacked adequate sensitivity, requiring the presence of 10^4-10^5 copies of the target nucleic acid. The development of PCR[69] and other nucleic acid amplification techniques overcame that sensitivity barrier and has led to the development of nucleic acid–based diagnostic tests for many viruses. Nucleic acid amplification tests (NAATs) were originally directed at viruses that were difficult or impossible to cultivate, viruses that grew very slowly in culture, and viruses for which antigen detection could not be applied because of antigenic diversity or because the level of viral antigen in clinical specimens was too low to permit successful detection. NAATs were also very advantageous for specimens such as CSF or ocular fluid for which sample volume could be limiting. Still another advantage of nucleic acid detection as a diagnostic method is the stability of DNA as an analyte, so that detection of viral nucleic acids can be done even when conditions of transport lead to loss of virus viability. Currently, NAATs are being applied to almost all viruses that are of interest in diagnostic virology.

Nucleic Acid Amplification Assays

Target Amplification

Polymerase chain reaction

PCR is the prototype of target amplification assays and employs short oligonucleotide primers and a thermostable DNA polymerase such as *Taq* polymerase to amplify a segment of target DNA that is typically 100-1000 base pairs (bp) in length. Classic PCR includes repetitive cycles, each

consisting of denaturation, primer annealing, and extension steps that take place at different temperatures. Progression through the steps of the cycle is controlled by a thermal cycler that controls the temperature of the reaction. After PCR amplification, the PCR product (also known as the *amplicon*) can be detected by gel electrophoresis or by one of several probe-hybridization techniques (eg, Southern blotting). The analytic sensitivity of PCR can be as low as 1-10 copies of target DNA. Because of its simplicity and broad applicability, PCR remains the most widely used NAAT.

Most PCR in diagnostic virology laboratories is carried out using real-time PCR in which reaction products are detected as they are synthesized.[24,33] Compared with conventional PCR, real-time PCR has several important advantages. Because the accumulation of PCR product is monitored in the reaction tube, no separate detection method (eg, gel electrophoresis) is required, thus shortening the effective assay time markedly and decreasing the risk of contamination of the laboratory environment by the amplified PCR product. The time from setting up the assay to completion can be <1 hour. Many systems allow the performance of multiplex reactions with simultaneous amplification and detection of more than one product. Of great importance, by following the emergence of detectable PCR amplicon vs the number of PCR cycles, real-time PCR allows estimation of the amount of viral nucleic acid within a clinical sample. Alternatively, absolute quantitation of viral nucleic acids can be performed using digital PCR techniques, which partitions the PCR reaction spatially or within emulsions into tens of thousands of separate PCR reactions.

Detection of PCR products with real-time PCR can be achieved through various methods. The simplest system uses the DNA binding dye SYBR Green, which emits fluorescence when it is bound to double-stranded DNA (dsDNA). When SYBR Green is included in a PCR reaction, the intensity of fluorescence is proportional to the amount of PCR product. Because SYBR Green binds nonspecifically to any dsDNA, signal is generated by undesired amplification products such as primer dimers, as well as by intended amplicons. Discrimination between different amplification products can be achieved through the use of dissociation curves, referred to as *melting point analysis*. Melting point analysis is performed after completion of the amplification reaction by recording fluorescence as the temperature of the reaction mix is gradually increased. When the dissociation temperature (melting point) of the dsDNA reaction product is reached, SYBR Green is released and fluorescence decreases. The melting point is affected by both length and sequence of the PCR product and thus is precisely defined for a specific PCR product. Unintended amplification products will usually have different melting points, allowing easy discrimination from the intended PCR product.

The other detection systems are based on the use of oligonucleotide probes that are labeled with fluorescent dyes that interact with one another according to principles of fluorescence resonance energy transfer (FRET). The main assay format used is the fluorogenic 5′ exonuclease assay (also referred to as the *Taqman assay*). This assay uses a probe that is complementary to a segment of the intended PCR product located between the PCR primers. This probe is labeled with two fluorescent dyes, one called the *reporter* that is linked to the 5′ end of the probe, and the other called the *quencher* that is linked to the 3′ end. When they are in close proximity (ie, bound to opposite ends of an oligonucleotide probe), the quencher prevents fluorescence by the reporter. During the extension step of PCR, the probe labeled with both dyes binds to the PCR product as it is being synthesized. During extension, the 5′ exonuclease activity of *Taq* polymerase cleaves nucleotides from the 5′ end of the bound probe, releasing the reporter dye away from the quencher, thus allowing it to emit light on excitation. The intensity of fluorescence is proportional to the amount of PCR product.

The hybridization probe assay format uses two oligonucleotide probes that are homologous to adjacent portions of one of the strands of the amplified DNA. The probes are chosen so that the 5′ end of one probe is within a few nucleotides of the 3′ end of the other probe. These adjacent ends are each labeled with a fluorescent dye. The dye on the 3′ end is termed the *donor dye* and the dye on the 5′ end is termed the *acceptor dye*. The required property of these dyes is that when they are in close proximity (within a distance of several nucleotides), excitation of the donor dye leads to emission of light by the acceptor dye. When both probes bind to their target sequences, the two dyes are within the proximity required for excitation of the acceptor dye and fluorescence occurs. The fluorescence intensity again is proportional to the amount of PCR product. Alternatively, in the molecular beacon format, a hairpin probe self-hybridizes bringing the fluorophore in proximity to a quencher due, while hybridization of probe to the target nucleic acid separates the quencher from the fluorophore allowing signal detection.

Because *Taq* polymerase uses only DNA as a template, the use of PCR to detect viral RNA sequences requires the inclusion of a reverse-transcription (RT) step before PCR (RT-PCR). Special care must be used in specimen processing because of the susceptibility of RNA to digestion by ribonucleases that may be present in clinical samples. In addition to detecting virion RNA, RT-PCR can also be applied to the detection of viral messenger RNA. This may be particularly useful in the diagnosis of infection caused by viruses that have a latent phase in their life cycle. For these viruses, detection of viral DNA might not distinguish between latent and productive infection, whereas detection of a messenger RNA (mRNA) expressed only in productive infection would be evidence of active viral infection.[70] In addition, in the case of SARS-CoV-2 diagnostics, inclusion of multiple targets for detection in RT-qPCR assays has been especially helpful for both insuring against diagnostic failure due to future viral evolution with sequence changes as well as tracking specific viral strains if and when a target does fail.

RNA amplification assays

Other approaches have been developed that are directed at amplification of RNA. *Transcription-based amplification* (Fig. 16.5) uses three enzymes, RT, ribonuclease (RNase) H, and T7 RNA polymerase, to amplify a target RNA sequence, employing a series of reactions that mimic the retrovirus replication scheme. An advantage of transcription-based amplification assays is that they are isothermal and thus do not require complicated instrumentation. The assays begin with synthesis of a DNA strand that is complementary to the RNA target, using a primer that contains a T7 polymerase binding site at its 5′ end. The resulting DNA-RNA hybrid is converted to dsDNA by the action of RNase H and a second primer that also contains a 5′ T7 polymerase binding site. The dsDNA product then serves as a template for transcription driven by T7 RNA polymerase. The newly synthesized RNA transcripts serve as templates for additional cycles of the reaction. Variants of transcription-based amplification include transcription-mediated amplification (TMA),[23] self-sustained sequence replication (3SR),[32] and nucleic acid sequence–based amplification (NASBA).[14] Currently, NASBA assays for HIV, enteroviruses, and CMV pp67 have been approved by the FDA and are marketed by bioMérieux Inc. (Durham, NC) under the trade name NucliSENS. TMA is the basis for the Panther assays for HIV, HCV, HBV, SARS-Cov-2, influenza A, and influenza B marketed by Hologic (San Diego, CA).

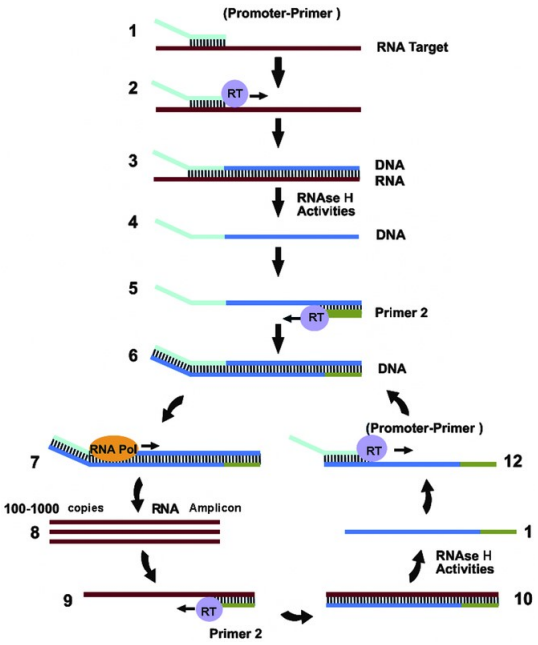
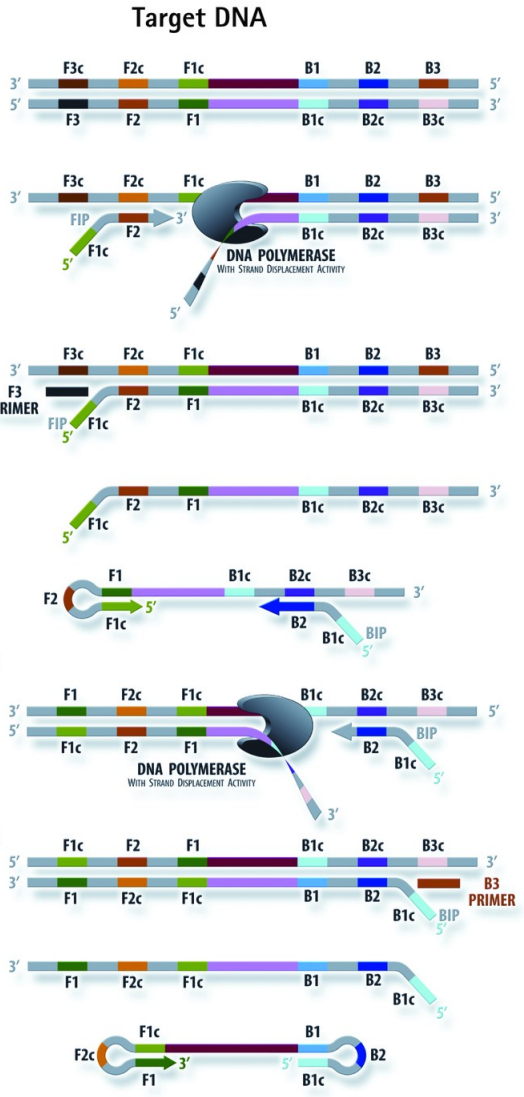


FIGURE 16.5 **Transcription-mediated assay, also called 3SR**. The 3SR reaction depends on a continuous cycle of reverse transcription and transcription reactions to replicate an RNA target by means of complementary DNA (cDNA) intermediates. Steps 1-6 depict the synthesis of a double-stranded cDNA, which is a transcription template for T7 RNA polymerase. Complete cDNA synthesis is dependent on the digestion of the RNA in the intermediate RNA-DNA hybrid (step 4) by ribonuclease (RNase) H. Transcription-competent cDNAs yield antisense RNA copies of the original target (step 7, right). These transcripts are converted to cDNA containing double-stranded promoters on both ends in an inverted repeat orientation (steps 7-12). These cDNAs can yield either sense or antisense RNA, which can re-enter the cycle.

The loop-mediated isothermal amplification (LAMP) (Fig. 16.6) is an amplification reaction that employs four primers and a strand-displacing DNA polymerase.[55] The inner primers have sense and antisense sequences. When they initiate DNA synthesis, the result is a stem-loop structure, which itself serves as a target for additional amplification. The resulting reaction can produce 10^9 copies of target DNA or RNA in <1 hour under isothermal conditions. The reaction can be monitored by measuring turbidity, which results from the accumulation of magnesium pyrophosphate, a by-product of the reaction. Alternatively, the synthesis of dsDNA can be measured using an agent such as SYBR Green, which generates fluorescence when it intercalates between the strands of dsDNA. The reaction has high specificity by virtue of the inclusion of multiple primer sets. The isothermal nature of the reaction, which obviates the requirement for a thermal cycler, makes LAMP attractive for use as a point-of-care test or for use in the developing world. Addition of CRISPR-based detection to (RT-)LAMP-based amplification (or other amplification methods) allows for additional sequence-based specificity in the reaction to confirm that a given viral species was amplified.[7,38] Given the specificity of CRISPR hybridization and the numerous CRISPR-based nucleases now available, such genotyping can be performed in a highly parallel approach within a single reaction vessel. Other methods involving signal amplification are branched-chain DNA assays, hybrid capture assays, and invader assays (e-Figs. 16.2-16.4).

LAMP Process

1. Solution temperature at 60°–65° C
2. Forward Initiating Primer (FIP) anneals to Target Sequence
3. DNA Polymerase initiates synthesis that displaces single strand template DNA
4. Through polymerase activity, a strand complementary to the DNA template is formed
5. The F3 Primer anneals to the F3c Region
6. DNA Polymerase initiates synthesis and the FIP-linked complementary strand is replaced
7. This strand forms a "stem loop" at the 5' end due to complementary F1 and F1c regions
8. The BIP anneals to the 3' end of the "stem loop" strand
9. The B3 Primer anneals to the B3c target
10. From the 3' end, polymerase synthesizes a complementary DNA strand
11. DNA reverts from a Loop structure to a linear structure
12. The BIP linked complementary strand is displaced as a single strand
13. This strand forms stem-loops at either end due to the activity of the dual complementary primers
14. This is the starting structure for LAMP Cycling



A

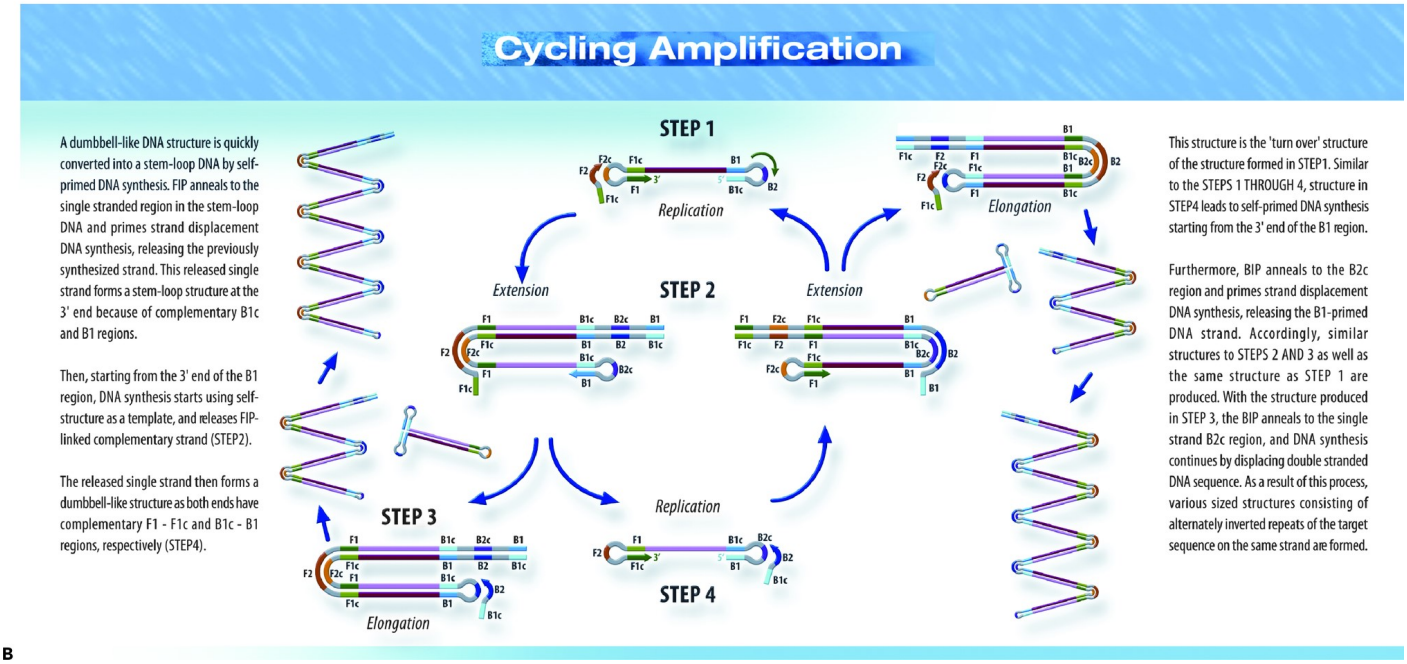
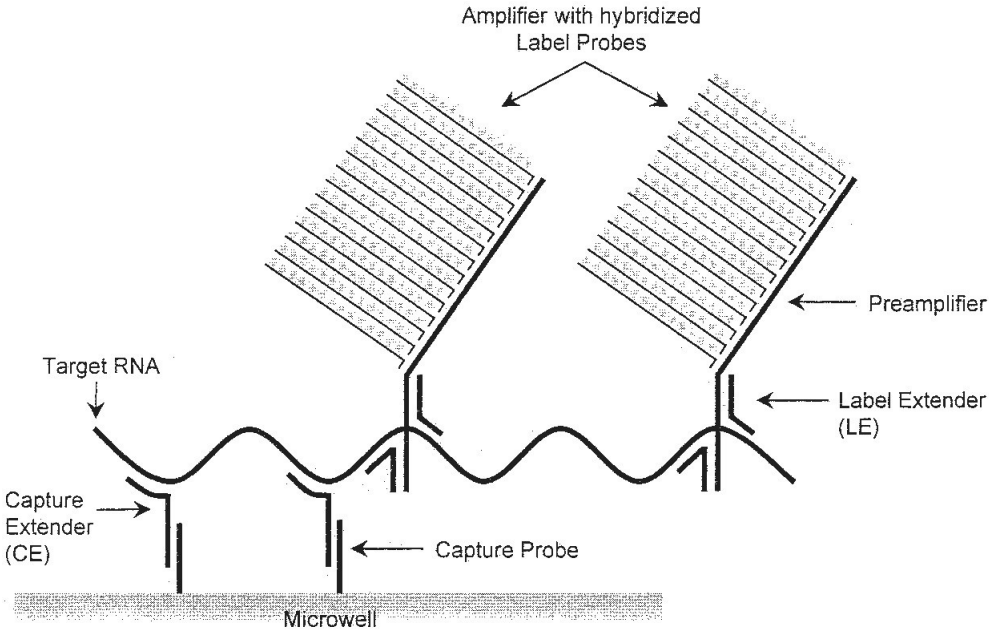
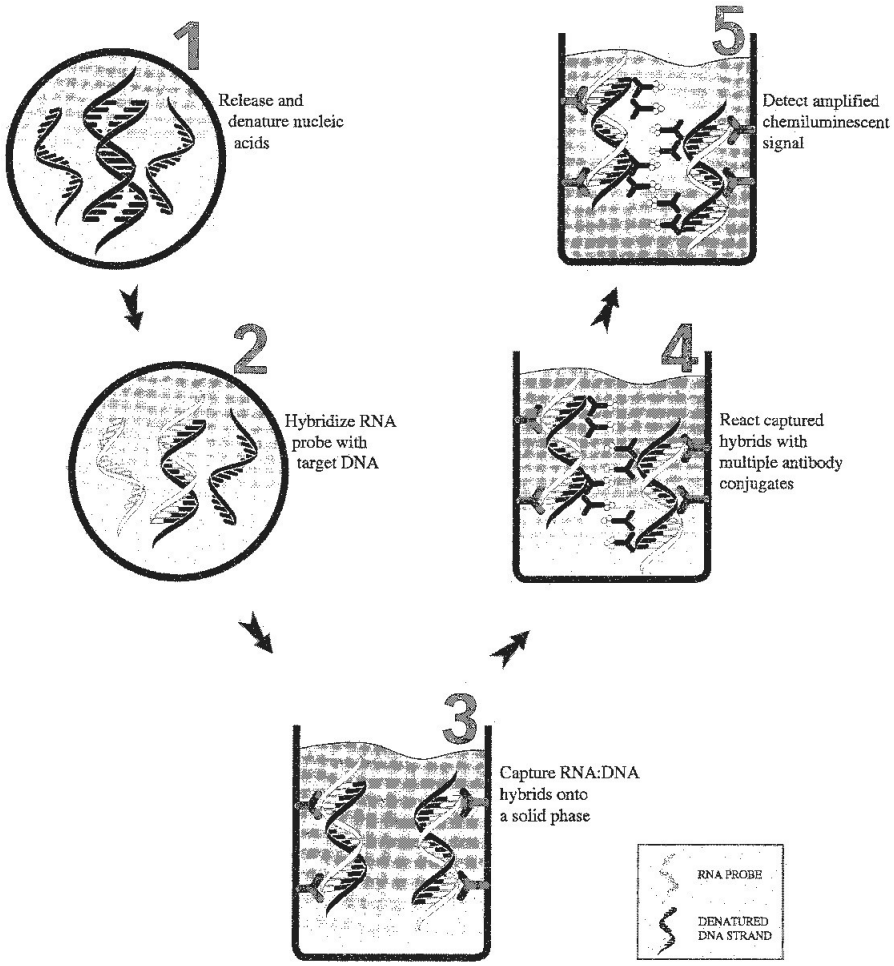


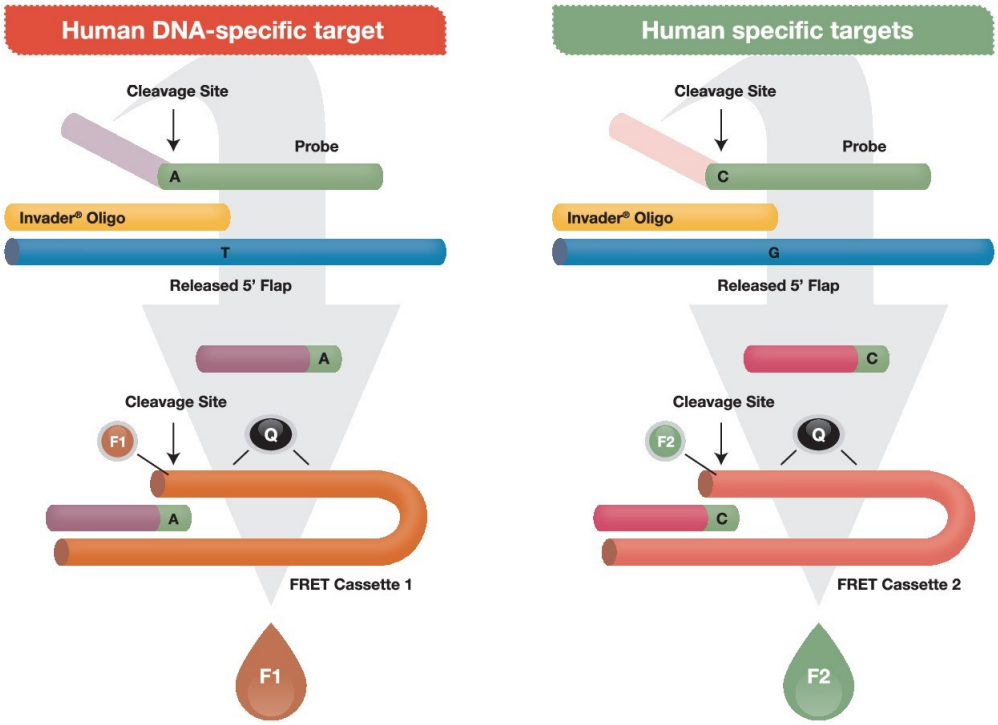
FIGURE 16.6 **Loop-mediated amplification reaction (LAMP assay)**. **A**. Steps leading up to the formation of the "dumbbell" structure. **B**. The cycling steps of the reaction, which lead to extremely rapid amplification. (Courtesy of Meridian Bioscience, Inc.)



e-FIGURE 16.2 **Branched-chain DNA assay as used for ultrasensitive detection of human immunodeficiency virus (HIV).** After liberation of viral nucleic acid from the clinical specimen by a lysis buffer, viral nucleic acid is hybridized with two sets of bifunctional oligonucleotide probes, each of which contains sequences complementary to the target. One set of probes also contains a generic sequence complementary to a capture probe that is bound to the surface of a microtiter tray and serves to bind the target to the solid surface. The other set of probes contains a sequence complementary to *preamplifier* molecules. Additional specificity is achieved because two of the target-specific probes must be juxtaposed in the correct orientation to stabilize the binding of the preamplifier molecule. Each preamplifier molecule binds numerous amplifier molecules, each of which binds many subsequently added alkaline phosphatase–labeled probes. A chemiluminescent substrate is added and generated light is read by a luminometer. The cascade effect results in amplification of the signal generated from initial binding of probes to the target and allows for detection and quantification of nucleic acid present in the specimen. (Courtesy of Bayer Diagnostics, Emeryville, California.)



e-FIGURE 16.3 **Hybrid capture assay.** In step 1, target DNA is released and denatured. In step 2, RNA probe hybridizes with target DNA. In step 3, RNA-DNA hybrids are captured by an antibody to RNA-DNA hybrids that is bound to the sides of the reaction vessel. In step 4, an enzyme-conjugated antibody that recognizes RNA-DNA hybrids binds to the hybrids. In step 5, a chemiluminescent substrate is added, and light is emitted if hybrids have been formed. (Courtesy of Digene Corporation, Beltsville, Maryland.)



e-FIGURE 16.4 **Invader assay.** The **left side** of the figure shows a positive control reaction for human histone 2DNA and the **right side** shows the reaction to detect human papillomavirus (HPV). For each reaction, the probe oligo and the Invader oligo bind to the

target, with a short region of overlap occurring at a targeted single nucleotide polymorphism (SNP). This results in a structure that is cleaved by the activity of an enzyme called "cleavase," releasing the 5′ end of the probe oligo at the position of overlap. The sequence of the released portion is homologous to the 3′ end of a third oligo, which includes a fluorophore (F1 for the human DNA reaction and F2 for the HPV reaction) and a quencher (Q) and is referred to in the figure as the FRET cassette. The hairpin configuration assumed by the FRET cassette brings the quencher sufficiently close to the fluorophore to allow its fluorescence to be quenched. Binding of the released portion of the probe oligo to the FRET cassette results in a region that is another substrate for cleavase. The activity of cleavase releases the fluorophore, resulting in generation of a fluorescent signal. (Courtesy of Hologic.)

Contamination

A general concern regarding highly sensitive nucleic acid amplification assays is the occurrence of false-positive findings resulting from contamination of the reaction with exogenous nucleic acids. The source of contaminating nucleic acids can be either amplified products from previous reactions or viral nucleic acids present in a different specimen, especially one containing a high level of the intended target. Prevention of contamination requires fastidious attention to procedural detail within the laboratory. A number of specific preventive techniques are widely used to prevent carryover contamination after PCR, including the use of positive displacement pipettes or plugged pipette tips to block aerosol production, UV irradiation of reaction components (not including *Taq* polymerase and PCR primers that can be inactivated by UV irradiation), use of small working aliquots, and frequent cleaning of equipment and laboratory surfaces with 10% bleach. An important precaution is the physical separation of the laboratory into sections where (1) the specimen is prepared, (2) the PCR reaction is set up, (3) the PCR amplification reaction takes place, and (4) the detection of amplification products is carried out. After PCR has taken place, the reaction tube is opened only in the section of the laboratory devoted to product detection. Techniques such as the inclusion of uracil-N-glycosylase or psoralen and isopsoralen derivatives in the reaction mixture were developed to minimize the danger of PCR contamination. Real-time PCR is less susceptible to contamination because product detection does not require manipulating the amplified product. For any form of nucleic acid testing, an additional concern is contamination from one specimen to another, which may occur during specimen handling in the laboratory. Inclusion of negative controls in the reaction setup is important in order to detect this form of contamination.

Nucleotide Sequencing

Sequencing of PCR amplification products can be carried out using the cycle-sequencing reaction or pyrosequencing. Sequence information can be used for several purposes including precise identification of a virus, genotyping, and the presence of mutations associated with antiviral drug resistance or unusual clinical manifestations. Genotypic resistance assays are performed most commonly for HIV, HCV, CMV, HBV, and influenza A virus (see section on Antiviral Susceptibility Testing). For HBV, nucleotide sequencing of the nucleocapsid gene and its associated promoter is also used for detection of core promoter and precore mutations, which have been associated with unusually severe disease and progression to chronic infection.[56] High-throughput ("next-generation") sequencing, as described further below, has important applications in diagnostic virology, including for antiviral susceptibility testing, for identification of viruses growing in cell culture that are not identifiable by conventional means,[76] and for discovery of known[10,86] and novel viruses.[13,57] Increasingly, viral sequencing is also used for epidemiological purposes such as tracking transmission for infection prevention or monitoring effectiveness of vaccines over time.[30,59]

Microarray Technology

High-density microarrays consist of hundreds or thousands of oligonucleotide probes bound to a solid phase, such as a small silicon chip or glass slide. Amplified nucleic acid can be hybridized to the array, and binding to specific probes can be identified. Use of probes representing all possible nucleotide sequence variations within a target sequence allows rapid determination of the nucleotide sequence.[31] Sequence variants present within the specimen can also be detected. An early application in diagnostic virology has been for rapid sequencing to detect HIV mutations associated with resistance to antiretroviral drugs.[44] In another application, sequences representing all sequenced viruses have been selected to create a microarray that can be used to discover unknown viruses. This microarray was successful in categorizing the agent of severe acute respiratory syndrome (SARS) as a new coronavirus.[83] The major application of microarray technology in diagnostic virology to date has been the use of liquid arrays, which are used for the simultaneous detection of multiple respiratory viruses, as exemplified by the xTAG RVP described later. Future uses of liquid arrays and other microarray technology under development include assays to detect multiple viruses (as well as other classes of pathogens) associated with gastroenteritis, sexually transmitted diseases, sepsis, and bioterrorism.

Commercial Nucleic Acid Amplification Platforms and Tests

Government regulation

In the United States, commercially marketed diagnostic tests and test platforms are regulated by the FDA. Before marketing a test for diagnostic use, a manufacturer must receive authorization from the FDA. This authorization can take one of several forms. If there is no similar test on the market, the manufacturer must apply for premarket approval (PMA). This is a rigorous and expensive process that requires the manufacturer to provide evidence for the accuracy and utility of the proposed test. A test that is "substantially equivalent" to a test that has been previously approved or cleared by the FDA is cleared for use through a mechanism referred to as 510K, after the section of the Federal Food, Drug, and Cosmetic Act that describes the process. Some diagnostic test materials are marketed under the classification "analyte-specific reagent (ASR)." This term is used by the FDA to refer to reagents or materials, including nucleic acid primers and probes, that must meet certain specifications set by the FDA, including being produced utilizing current "good manufacturing practices." During a public health emergency, such as that seen for SARS-CoV-2, the FDA authorizes diagnostic tests using an emergency use authorization pathway, which requires fewer studies than the typical 510K pathway. For instance, over 400 EUAs were issued by the FDA during the first 2 years of the SARS-CoV-2 public health emergency.

Individual laboratories may also develop their own assays that are intended for use only in that laboratory. These tests, referred to as "laboratory-developed tests" (sometimes also referred to as "home-brew assays"), are currently not actively regulated by the FDA but instead are under the jurisdiction of CLIA passed by the U.S. Congress in 1988. Laboratory-developed tests may use ASRs as components of the test and may also use reagents obtained from diverse sources not specifically labeled as ASRs. The performance of laboratory-developed tests must be verified according to specific criteria established by CLIA. Laboratory-developed tests filled an important niche for alternative specimen types and/or analytes for which the market is too small to garner commercial interest. In addition, laboratory developed tests were critical for pandemic preparedness such as allowing for early testing in the first months of the SARS-CoV-2 pandemic.[28]

Overview of commercial assays and platforms

Since the last edition of this book, there has been a dramatic increase in molecular diagnostic tests and test platforms that have been approved or cleared by the FDA for the detection of pathogenic viruses. No single platform permits the detection of all medically important viruses, so laboratories whose mission is broad-based viral diagnosis will need to employ multiple platforms. With the high demand for high-throughput molecular SARS-CoV-2 testing combined with increased preanalytical automation and workflow advantages along with increasing regulatory requirements, testing is increasingly carried out using commercial platforms vs laboratory-developed tests. Likewise, laboratories that provide different types of service will employ different platforms. For example, hospital-based laboratories may emphasize platforms that provide rapid turnaround time, commercial laboratories may employ platforms that allow high specimen throughput, and government laboratories may employ platforms that allow for testing of viruses of public health significance. The following paragraphs describe several categories of commercial molecular diagnostic tests.

Multiplex assays

Multiplex PCR refers to PCR reactions in which more than one primer set is incorporated into the reaction mix, allowing the detection of multiple targets. Multiplex assays may be developed either as commercial tests or as laboratory-developed tests. Currently, multiplex assays are being developed for important specimen types to amplify various viral and other microbial agents that are pathogenic at the specific body site. With the increasing availability of antivirals and need to test for viral, bacterial, fungal, and eukaryotic parasite targets at the same time, multiplex testing is increasingly the norm for a given specimen type and a significant proportion of diagnostic virology assays coming onto the market today.

The FilmArray Respiratory Panel was originally cleared by the FDA in 2011 for multiplex detection in nasopharyngeal swabs. The panel has undergone several updates since and detects influenza A and B viruses; influenza A subtypes H1, H1 2009, and H3; RSV; human metapneumovirus; adenovirus; parainfluenza viruses 1 through 4; rhinovirus/enterovirus; and SARS-CoV-2 and seasonal human coronaviruses NL63, HKU1, 229E, and OC43.[63] Several bacterial species (*Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Bordetella pertussis*, and *Bordetella parapertussis*) are also included. Extraction, amplification, and detection are carried out in a self-contained unit referred to as a "pouch." Nucleic acid extraction is accomplished using magnetic beads. The nucleic acid amplification that follows is a multiplex nested PCR assay. The first amplification reaction is a multiplex reaction containing outer primers for each target virus. The reaction mix is then diluted and combined with a new primer-free master mix that contains the fluorescent dye LC Green Plus, which binds to dsDNA. This mix is then moved by microfluidic techniques into an array of individual wells, each of which contains inner primers specific for one target virus. Each target is represented in at least three wells. Production of viral amplicons is confirmed by analyzing melting curves. The entire process from inoculation of sample to availability of results takes ~1 hour, with only 2 minutes of hands-on time. The manufacturer uses a similar approach for other syndromic testing panels. Broad-based sample-to-answer multiplex testing for respiratory viruses is also available via the xTAG Respiratory Virus Panel (Diasorin/Luminex), the eSensor or ePlex (Roche/GenMarkDx), and the QIAstat-Dx systems (Qiagen).

Real-time assays for multiple seasonal respiratory viruses (eg, influenza/RSV) exemplified by the Simplexa Flu A/B & RSV Test and Verigene RV+ (Diasorin) use separate primer pairs to simultaneously amplify influenza A and B viruses and RSV plus an internal control. The Simplexa assay is run on the 3M Integrated Cycler, an innovative instrument produced by 3M/Diasorin. Multiple different specimens can be loaded on the instrument and multiple assays can be run simultaneously. The Verigene RV assay uses an innovative system to detect amplified products in which the amplicons are captured by capture probes that are bound to specific sites on a microarray. Captured amplicons are then detected by binding of silver-coated gold nanospheres that have been tagged with oligonucleotides that are specific for the amplicons and are visualized by light scatter.

Integrated platforms

The term *integrated platform* refers to test platforms in which nucleic acid extraction, amplification, and product detection are carried out within the same device or instrument. Colloquially, integrated platforms are also called "sample-to-answer" platforms. These platforms represent an important advance in diagnostic microbiology because their simplicity allows them to be performed in many laboratories that would not otherwise be able to provide viral diagnostic testing. In fact, they are so simple that they could be performed in a physician's office and some models are CLIA waived, allowing testing outside of laboratories approved for "high-complexity" testing. A widely used integrated platform is the GeneXpert Real-time PCR system, marketed by Cepheid (Sunnyvale, CA). This platform employs test cartridges into which a specimen is inoculated. The cartridge is inserted into the GeneXpert instrument, which performs nucleic acid extraction, amplification, and product detection without any intervention by the user. A similar integrated system is the Diasorin Simplexa. Tests on the small integrated platforms generally require <2 hours to result with minimal hands-on time. Other higher-throughput quantitative integrated systems include the Hologic Panther Fusion, Roche cobas, and Abbott Alinity m, which allow for up to one thousand test results per day and provide results in <4 hours, also with minimal hands-on time. These systems are generally used for high-throughput testing of HIV, HBV, HCV, HPV, and SARS-CoV-2 but also allow for testing for transplant-associated viruses such as CMV and BK virus, respiratory virus panels, and STI testing.

Serology

Measurement of antiviral antibodies was one of the first methods used for the specific diagnosis of viral infections, and it remains an important tool in the diagnostic virology laboratory. The role of serology can be for the diagnosis of acute or current infection or for the determination of past infection with specific viruses, which may be helpful in assessing virus-specific immunity. Serologic diagnosis is important for viruses that are not detected readily by molecular assays or culture. Viruses for which serology is useful in diagnosing acute infection are shown in Table 16.4. Serology is uniquely useful for defining past infection with respect to specific viruses. It is important to use sensitive assays such as EIA for this purpose, because antibody levels indicative of past infection can decline to very low levels years after the infection. The specific assay used must also be selected with care to ensure that it measures antibodies that correlate with immunity. Viruses for which serologic testing is used to determine past infection/immune status are shown in Table 16.5.

TABLE 16.4 Serology for diagnosis of acute viral infection

Epstein-Barr[a]
Cytomegalovirus (mononucleosis syndrome)[a]
Hepatitis viruses (A-E)[b]
Measles[a]
Rubella[a]
Mumps[a]
Parvovirus B19[a]
Encephalitis viruses (eg, West Nile virus)[a]
Rabies
Hemorrhagic fever viruses[a]
Dengue, zika, and chikungunya[a]
Human immunodeficiency virus (HIV)
Human T-cell lymphoma or leukemia virus types 1/2 (HTLV-1/2)

[a]Virus-specific immunoglobulin M (IgM) assays are used.

[b]Virus-specific IgM assays are used for hepatitis A, B, and E.

TABLE 16.5 Serology for diagnosis of prior viral infection

Varicella-zoster virus
Herpes simplex virus
Cytomegalovirus
Epstein-Barr virus (immunoglobulin G antibodies to the viral capsid antigen)
Human herpesvirus type 6
Rubella
Measles
Mumps
Parvovirus B19
Hepatitis A virus (total antibodies)
Hepatitis B virus (anti-HBs)
Hepatitis E virus
SARS-CoV-2

Kinetics of the Antibody Response

Serologic diagnosis is based on the kinetics of the antibody response to viral infection. Virus-specific antibodies are absent in an individual who has never been infected with that virus, but typically become detectable several weeks after the onset of infection. Virus-specific IgM antibodies are often detectable for a short period before virus-specific IgG antibodies. The IgM response tends to decline within ~1-2 months, although low levels can persist for 1 year or more in some viral infections. IgG antibodies are much more long-lasting and can persist for the life span of the individual. Thus, the absence of virus-specific IgG and IgM antibodies signifies susceptibility to infection; the presence of virus-specific IgM antibodies, with or without virus-specific IgG antibodies, signifies current or very recent infection; and the presence of virus-specific IgG but not IgM antibodies signifies past infection. The latter pattern often, but not always, indicates immunity to subsequent infection.

An alternative serologic approach to the diagnosis of recent infection is based on the concept that the affinity of antigen-specific antibodies increases as the immune response matures.[17] Thus, antibodies produced shortly after infection have low affinity for the causative agent, and antibodies produced late after infection have high affinity. Detection of low-affinity antibodies has been used to diagnose recent infection with CMV,[16] rubella,[67] and HIV.[75]

The relationship between the onset of clinical manifestations of infection and the presence of virus-specific antibodies depends on the

incubation period of the infection. In infections with short incubation periods (eg, acute respiratory infections), antibodies are absent at the onset of symptoms. In viruses with incubation periods of 1 month or longer, however, virus-specific IgG and IgM antibodies are typically present at the onset of symptoms.

Serology in Reinfection and Reactivation

The serologic diagnoses of reinfection and reactivated infection are more complicated than that of initial infection. In both of these cases, the infection occurs in an individual with preexisting, virus-specific IgG antibodies. In reinfection, an anamnestic response may occur that results in an increase in the level of virus-specific IgG antibodies with continued absence of virus-specific IgM antibodies. Reactivation of latent infection, for example, in the case of some herpesvirus infections, may result in the appearance of virus-specific IgM as well as IgG antibodies.

Serology in Chronic Infections

The interpretation of serology is unique for viruses that typically cause chronic infection (eg, HIV) and the related retroviruses human T-cell lymphoma or leukemia virus (HTLV) types 1 and 2. For these agents, the detection of antibodies of any isotype to these viruses (in the absence of artificial immunization) virtually always signifies current infection. In the case of HCV, the confirmed presence of antibodies to the virus corresponds to active infection in ~85% of individuals. For HIV, assays that simultaneously measure HIV p24 antigen as well as HIV antibodies have proven useful because based on the early appearance of the p24 antigen, the combined test decreases the “window” during which a recently infected individual is serologically negative but capable of transmitting infection.[58]

Serologic Assays

Binding assays

The most widely used assays are those that directly measure binding of antibodies to viral antigens. Examples of binding assays include EIA and the indirect immunofluorescent antibody assay (IFA). Binding assays can be used in many different formats. In one common format, a viral antigen, which can consist of virally infected cells, a purified viral preparation, or a recombinant viral protein, is attached to a solid surface such as the inner surface of the well of a microtiter tray, a plastic bead, or a microscope slide in the case of IFA. Serum is added, allowing the binding of antiviral antibodies that might be present in the serum. After an appropriate incubation period, the serum is removed and the well is extensively washed. The next step is the addition of a second antibody (also referred to as a detector antibody) with specificity for human immunoglobulins. The second antibody is typically a mouse monoclonal or a polyclonal antibody from a nonhuman species. It is linked to an enzyme such as horseradish peroxidase. The second antibody binds to any human antibody present (ideally, because of specific binding to the viral antigen). The well is again extensively washed, and the presence of the second antibody is detected based on a colorimetric, radiometric, or fluorescent signal. An important advantage of binding assays is that they can be modified to detect IgM- or IgA-specific antiviral antibodies through the use of isotype-specific detector antibodies.

Immunobinding assays

The western blot and related recombinant immunobinding assays are assays that allow the identification of antibodies to specific viral proteins. In the western blot assay, viral antigens present in infected cells are denatured, separated by gel electrophoresis, transferred to a membrane, and allowed to react with the serum specimen. Binding of antiviral antibodies is detected through the use of labeled detector antibodies. The western blot is cumbersome but provides a very high level of specificity because of the ability to identify binding to individual viral proteins and the production of a characteristic blot fingerprint of a specific molecular weight. Thus, its main application in diagnostic laboratories is the confirmation of screening assays (eg, EIA) that are less specific. For example, for many years, the western blot was widely used to confirm a positive HIV EIA and may still be used to confirm HSV serological results. The recombinant immunobinding assay differs from the western blot in that specific viral antigens are produced in *in vitro* expression systems and artificially attached to membranes in a predetermined configuration, often in dots or in bands. The membranes are then incubated with serum, and bound antibodies are detected, as in the western blot. Because the antigens used in the immunobinding assay are not denatured as they are in routine western blots, these assays may have advantages in detection of antibodies to conformational epitopes.

Functional assays

Functional assays are based on detection of specific activities resulting from the binding of specific antibodies to viral antigens. An important example is the neutralization assay, which measures the ability of antibodies to block viral infectivity. In neutralization assays, serial dilutions of the serum to be tested are incubated with a standardized quantity of infectious virus. After a short incubation period, the serum-virus mixtures are added to cells that support the growth of the virus, in parallel with a similar inoculum of virus that has not been incubated with serum. The neutralizing antibody titer is the highest serum dilution that prevents infection of the cells. The neutralization assay correlates well with protection from infection and is sometimes considered the standard against which other serologic assays should be measured. Because it is cumbersome and expensive, the neutralization assay is rarely used in routine diagnostic laboratories, but it is commonly seen in vaccine clinical trials as a primary or secondary outcome measure. Other examples of functional assays are the hemagglutination-inhibition assay, in which the presence of antiviral antibodies is detected by their ability to block virus-induced hemagglutination, and the complement fixation assay, which measures the ability of antiviral antibodies to fix complement, preventing the complement from lysing indicator erythrocytes. These assays have been largely replaced by binding assays.

Agglutination assays

Viral antigens can be bound to a variety of particles, including fixed erythrocytes and latex particles. Agglutination assays are performed by mixing dilutions of serum with a suspension of antigen-coated particles. The agglutination titer is the highest serum dilution that results in visible agglutination of particles. The advantage of agglutination assays is simplicity; they are rapid and do not require sophisticated equipment. Therefore, they are well suited for *stat* testing and for use under field conditions. For example, a rapid latex agglutination assay for varicella antibodies can be used for rapid determination of the varicella immune status of health care personnel who are exposed to a patient with varicella.

Virus-Specific IgM Assays

Assays for virus-specific IgM antibodies are important because they often allow for diagnosis of an acute infection based on analysis of a single serum specimen obtained during the acute phase of illness. These assays are most likely to be useful in diseases with incubation periods that are sufficiently long so that virus-specific IgM levels are present at detectable levels at the onset of illness. Hepatitis A provides an excellent example; because of its incubation period of 2-6 weeks, hepatitis A IgM antibodies are virtually always detectable at the time patients seek medical care.

A number of methods are used to measure virus-specific IgM antibodies. Two older methods include (1) separating IgG and IgM fractions before performing the virus-specific antibody assay and (2) performing the virus-specific assay before and after procedures, such as treatment with β-mercaptoethanol, that selectively destroy IgM antibodies. Many modern virus-specific IgM antibody assays use IFA or EIA with a detector antibody that binds only to human IgM. The IgM capture assay is an alternative approach in which IgM antibodies present in the serum sample being tested are first *captured* using an antibody specific for human IgM. A preparation of viral antigen is added, followed by an enzyme-labeled antibody specific for the viral antigen. Binding is detected by the addition of the appropriate enzyme substrate.

Virus-specific IgM assays have several potential pitfalls and must be interpreted in conjunction with clinical findings. False-positive results can occur if the serum being tested contains rheumatoid factor (IgM antibodies to human IgG) plus virus-specific IgG antibodies that form complexes that may be falsely detected by the assay as virus-specific IgM antibodies. This problem can be avoided by pretreatment of the serum to remove either rheumatoid factor or IgG antibodies. One or the other of these pretreatments is included routinely in many commercial virus-specific IgM antibody assays. False-negative results can occur if the serum being tested contains high levels of virus-specific IgG antibodies that may compete with the virus-specific IgM antibodies. The IgM-capture assay configuration avoids this problem, as does serum pretreatment to remove IgG.

Complexities in the nature of the IgM antibody response must also be considered in interpreting virus-specific IgM antibody tests. In some patients, the IgM antibody response is transient or low level, leading to failure to detect virus-specific IgM antibodies, whereas in others, low levels of IgM antibodies may persist for 1 year or longer, decreasing the specificity of the assay as an indicator of very recent infection. In herpesvirus infections, IgM antibodies can sometimes be detected in reactivations as well as in primary infection.

Saliva and Urine Assays

Virus-specific antibodies can often be detected in saliva and urine. These body fluids are attractive for use in serologic assays because they avoid the need for phlebotomy. Assays for HIV antibodies in these fluids are commercially available and have performance characteristics similar to assays carried out on serum.[20,79]

Cerebrospinal Fluid Serology

Serologic testing can be applied to CSF for the diagnosis of central nervous system (CNS) infection. For unusual viruses (eg, rabies), the presence of any virus-specific antibodies within the CSF is diagnostic of active infection. For the diagnosis of encephalitis caused by the alphaviruses, bunyaviruses, or flaviviruses, the presence of virus-specific antibodies in CSF is highly suspicious and the presence of virus-specific IgM antibodies in CSF is diagnostic and often more clinically sensitive than CSF PCR. For more common viruses (eg, herpesviruses) or the common respiratory viruses, the mere presence of virus-specific antibodies in CSF is not diagnostic of CNS infection because antibodies produced in the blood are present in the CSF even in the absence of CNS infection. The problem is further complicated by the fact that defects in the blood-brain barrier that can occur during many neurologic diseases can increase the passage of antibodies from blood to CSF. Therefore, for the common viruses, intrathecal synthesis of specific antiviral antibodies must be demonstrated to provide evidence of CNS infection.

Intrathecal synthesis of a specific antiviral antibody is evaluated by determining the quotient of two ratios: (1) the ratio of specific antiviral antibody level in CSF to the level in serum and (2) the ratio of total IgG in CSF to total IgG in serum ($CSF_{specific\ antibody}$:$serum_{specific\ antibody}$ divided by CSF_{IgG}:$serum_{IgG}$). A quotient >1.5 is evidence of intrathecal antibody synthesis of the specific antibody. The antibody assay used to measure the specific antiviral antibody must have certain special characteristics. First, it must be very sensitive, because CSF antibody levels are usually low (~1000 times less than serum levels). Second, it must be capable of providing a quantitative result that allows calculation of the CSF-to-serum ratio of specific antibody level. EIA is usually used, but testing of a series of dilutions of CSF and serum is required to obtain linear quantitative estimates of antibody level suitable for calculating the necessary ratio. Because of the complexities involved, accurate determination of intrathecal antibody synthesis of specific antiviral antibodies is performed in only a limited number of reference laboratories.

Measurement of intrathecal antibody synthesis has been used in a variety of CNS infections. The applications for which experience is most extensive are encephalitis caused by HSV and VZV. In HSV encephalitis, the utility of determinations of intrathecal antibody synthesis is limited because intrathecal antibody synthesis may not be detectable until 7-10 days after the onset of illness. Interestingly, intrathecal antibody synthesis can persist for years after the acute infection. Therefore, the main application is in cases in which specimens were not available early in the illness to allow a definitive diagnosis based on PCR. A caveat concerning diagnosis by demonstration of intrathecal antibody synthesis is that nonspecific polyclonal intrathecal antibody synthesis can occur in some diseases (eg, multiple sclerosis).[65] This pitfall can be avoided by evaluating intrathecal synthesis of antibodies to at least one other virus other than the one of primary interest. Serologic diagnosis has been shown to be more sensitive than PCR for diagnosis of VZV vasculopathy.[52] This is explained partly by the fact that CSF samples from some patients in this study were obtained months after the onset of neurologic manifestations.

Antiviral Susceptibility Testing

In recent years, antiviral drugs have been licensed for the treatment of infections caused by HSV, VZV, CMV, influenza A and B, RSV, SARS-CoV-2, HIV, HCV, and HBV. Not surprisingly, increasing use of these drugs has been accompanied by the appearance of antiviral drug resistance. Testing for resistance may be required for optimal patient management. Antiviral drug susceptibility testing is performed in some diagnostic virology laboratories and is also available through reference laboratories.

Phenotypic Assays

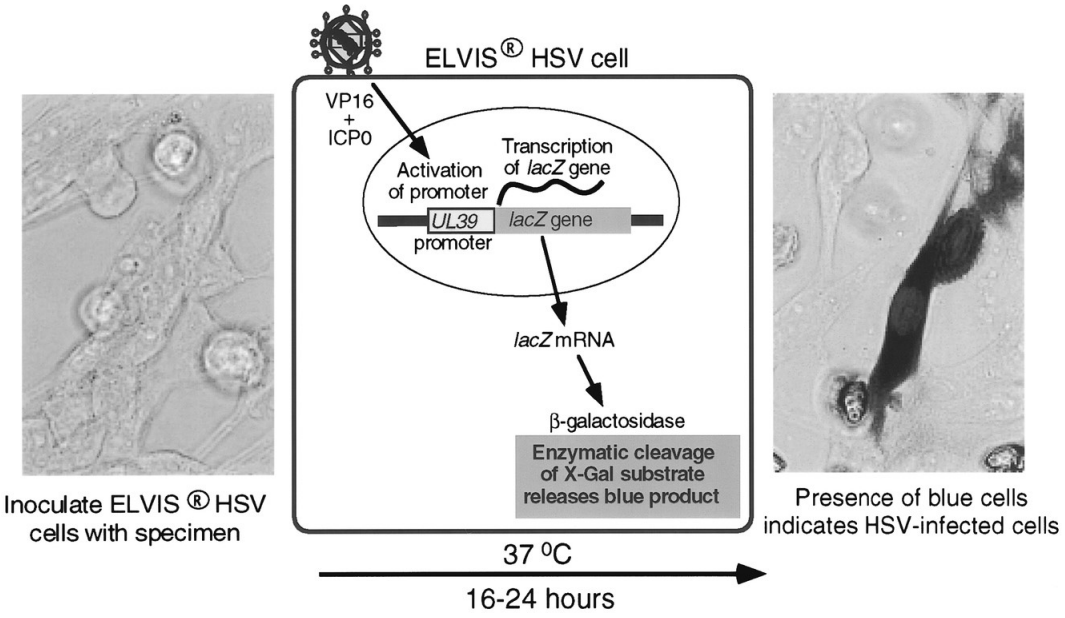
Antiviral susceptibility testing is divided into phenotypic and genotypic methods. Phenotypic methods analyze the effect of antiviral drugs on the

replication of virus, which can be measured by infectivity, viral antigen or nucleic acid production, or effect on the activity of a viral enzyme such as influenza neuraminidase. Phenotypic assays are important because they directly measure the effects of antiviral drugs on viral replication. In addition, they are more universal than genotypic assays because they can be used when the genetic basis for resistance is unknown. In the case of HIV, phenotypic assays are useful in analyzing viruses in which the presence of multiple mutations makes it difficult to predict phenotype from genotypic data. Finally, phenotypic assays must be used to determine the significance of candidate mutations found in drug-resistant isolates. However, from a practical standpoint, phenotypic assays tend to be labor-intensive, slow, and poorly standardized. In addition, there is a theoretical concern that the growth of the virus in cell culture may lead to selection of nonrepresentative viral variants, especially because antiviral resistance may in some instances be associated with decreased viral fitness. For these reasons, phenotypic assays are not performed in most diagnostic virology laboratories. Table 16.6 shows recommended *cutoffs* that define resistance to several drugs used for HSV and CMV infections.

TABLE 16.6 Cutoffs for antiviral susceptibility testing for herpes simplex virus (HSV) and cytomegalovirus (CMV) using the plaque reduction assay

		Cutoff (μM)		
Virus (Reference)	**Drug**	**Sensitive**	**Intermediate**	**Resistant**
HSV[68]	Acyclovir	<8		≥8
	Foscarnet	<330		≥330
CMV[18,85]	Ganciclovir	≤6	6-12	>12
	Foscarnet	≤400		>400
	Cidofovir	≤2		>4

The plaque assay was the traditional method for performing phenotypic susceptibility testing. However, this method is rarely used in diagnostic virology laboratories for the reasons mentioned previously and will not be discussed further here. Assays measuring viral antigen or nucleic acid production are alternatives to infectivity-based assays. A variety of assays have been used in individual laboratories, but they are not commercially available. The ELVIS assay has also been adapted for the performance of antiviral susceptibility testing (e-Fig. 16.5).[74,77] For influenza virus, resistance to neuraminidase inhibitors has been measured using neuraminidase inhibition assays based on either fluorescent or chemiluminescent signal generation.[8,53]



e-FIGURE 16.5 **ELVIS herpes simplex virus (HSV), a genetically engineered cell line for the detection of HSV clinical samples.** ELVIS cells are baby hamster kidney (BHK) cells that contain a β-galactosidase gene under the control of the HSV promoter UL39. If HSV is present in the specimen, viral particles enter the cells and the HSV proteins ICP0 and VP16 activate the UL39 promoter, leading to synthesis of β-galactosidase. The presence of β-galactosidase is detected by adding X-gal, a substrate for the enzyme. The activity of β-galactosidase on X-gal results in blue staining of ELVIS cells, visible microscopically.

Recombinant phenotypic assays have been developed and widely used for HIV. These assays involve insertion of HIV genes that are targets for antiretroviral drugs (polymerase, protease, integrase) from a patient being evaluated into a vector consisting of a rapidly replicating laboratory strain of HIV that also contains a reporter gene (eg, luciferase) that is used to measure viral growth.[60] These assays allow relatively rapid testing of the susceptibility of HIV to multiple drugs as well as to drug combinations. Commercial tests have been developed by Monogram Biosciences (San Francisco, CA) and Virco (Antwerp, Belgium) and are available in the United States through national reference laboratories. A special case is the HIV drug maraviroc, which blocks HIV entry by binding to the CCR5 receptor. Viruses that use the CXCR4 receptor rather than the CCR5 receptor are resistant to maraviroc. A commercial assay called Trofile (Monogram Biosciences)[85] is used to predict response to maraviroc by determining the receptor tropism of a patient's isolate.

Genotypic Assays

Genotypic assays test for the genetic basis for resistant phenotypes. A variety of methods are used, including detection of restriction fragment length polymorphisms, PCR assays that detect mutations by virtue of differential primer or probe binding, and nucleotide sequencing. With rapid improvements in sequencing technology, sequencing-based methods are becoming increasingly used, though it is difficult to turn around results fast enough for acute infections. Genotypic assays are informative only when the genetic basis for resistance is known and are most useful when the resistance being evaluated is accounted for by a limited number of genetic changes. During the SARS-CoV-2 pandemic, genotypic surveillance was used to inform the shipment of specific therapeutic monoclonal antibodies to different regions based on the prevalence of specific viral variants in the population.

Genotypic assays have been most widely used for CMV, HIV, HCV, HBV, SARS-CoV-2, and influenza A virus, because phenotypic assays for these viruses are slow and expensive or not widely available. Application of genotypic assays requires an extensive body of knowledge concerning the effect of specific mutations on antiviral resistance. Some single mutations may confer high-level resistance, while others may confer only partial resistance, and others are not associated with resistance at all.

For CMV, resistance to the currently FDA-approved drugs ganciclovir, foscarnet, maribavir, and cidofovir has been related to mutations in the phosphotransferase (*UL97*) and polymerase (*UL54*) genes while resistance to letermovir is associated with terminase (*UL56*) mutations.[11,46] Most laboratories now use sequencing to detect these mutations. Because ganciclovir-resistance mutations generally arise first in the *UL97* gene, some laboratories sequence this gene first, proceeding to sequencing *UL54* only if no significant mutations are detected in *UL97*, or if the patient is being treated with foscarnet or cidofovir, which exert their antiviral effect by inhibiting this gene. Different mutations within *UL97* or *UL54* are associated with different levels of antiviral resistance.[46]

For HIV, genotypic testing for resistance is commonly used by physicians in the developed world in the management of antiretroviral drug therapy. This testing involves RT-PCR amplification of regions of the polymerase and protease genes and sometimes the integrase gene, followed by nucleotide sequencing of the amplicons. Certain mutations are highly associated with phenotypic resistance and drug failure, whereas the significance of other mutations found in drug-resistant strains is less clear. Interpretation of HIV genotypic testing is most straightforward in patients who are not highly drug experienced and have relatively small numbers of mutations. Patients who have been treated more extensively may have multiple interacting mutations and may also have mutations that lie outside of the segments that are sequenced but nevertheless affect susceptibility to antiretroviral drugs.[26] High-throughput sequencing can be used to detect rare drug-resistant mutants that make up a small percentage of viral genomes.[73] The clinical significance of small minority populations of drug-resistant mutants is still under investigation.[6,12]

The presence of resistance of HBV to antiviral agents is detected by sequencing of the DNA polymerase gene. Genotypic testing for HSV has been less commonly used than phenotypic testing because of the diverse genetic loci within the HSV thymidine kinase and polymerase genes that can account for resistance, but as less and less viral isolation is performed genotyping testing is likely to increase in frequency.[21]

Selected Clinical Problems

e-Tables 16.4 through 16.9 provide information related to specific areas of diagnostic virology not covered elsewhere in the chapter.

e-TABLE 16.4 Laboratory diagnosis of human herpesvirus infections

Virus	Clinical Problem	Test(s)	Specimen
Herpes simplex	Mucocutaneous lesions	Culture, NAAT, FA stain	Swab of lesion
	Encephalitis or meningitis	NAAT	CSF
Varicella-zoster	Mucocutaneous lesions	Culture, NAAT, FA stain	Swab of lesion
	Encephalitis or meningitis	NAAT	CSF
	CNS vasculopathy	Intrathecal antibody synthesis, NAAT[a]	Serum or plasma plus CSF
Cytomegalovirus	Systemic infection in immunocompromised individual	Quantitative NAAT, pp65 antigenemia assay	Plasma or whole blood
	Mononucleosis syndrome in nonimmunocompromised individual	CMV IgM antibody assay, NAAT	Serum or plasma for antibody assay, plasma or whole blood for NAAT
	Tissue-invasive disease (eg, involving the gastrointestinal or respiratory tract)	Histology, immunohistochemistry *in situ* hybridization, culture, NAAT	Tissue from involved organ
	Encephalitis	NAAT	CSF
	Retinitis	NAAT	Vitreous or aqueous fluid
Epstein-Barr virus (EBV)	Infectious mononucleosis	Heterophile antibody assay,[b] IgM antibodies to the viral capsid antigen	Serum or plasma
	Posttransplant lymphoproliferative disorder	Quantitative NAAT	Whole blood or plasma
	Primary central nervous system lymphoma	NAAT	CSF
	Encephalitis or meningitis	NAAT[c]	CSF[c]
Human herpesviruses types 6 (HHV-6) and 7 (HHV-7)	Acute infection[d]	NAAT,[e] IgM antibody assay, IgG avidity assay[g]	Plasma (NAAT), plasma or serum for IgM antibody assay, or IgG avidity assay[g]
	Systemic infection in immunocompromised individual	NAAT[e]	Whole blood or plasma[e]
	Encephalitis	NAAT[e]	CSF
Human herpesvirus type 8 (HHV-8) (Kaposi sarcoma virus)	Systemic infection[f]	NAAT	Blood or body fluid

[a]Intrathecal antibody synthesis has been shown to be more sensitive than NAAT for diagnosis of varicella-zoster virus central nervous system vasculopathy. (Nagel MA, Cohrs RJ, Mahalingam R, et al. The varicella zoster virus vasculopathies: clinical, CSF, imaging, and virologic features. *Neurology*. 2008;70:853-860).

[b]Slide tests or other modified heterophile assays are usually used.

[c]Should be performed only in the setting of primary EBV infection. In other clinical settings, specificity and positive predictive values are low.

[d]Some but not all cases have a rash and are diagnosed as roseola.

[e]Approximately 1% of the population has chromosomal integration of HHV-6 and has very high levels of HHV-6 DNA in blood and tissue, which is not indicative of disease attributable to HHV-6. Chromosomal integration in immunocompetent patients can be excluded by testing a follow-up sample (those with chromosomal integration will have persistently high levels of HHV-6 DNA) or a pretransplant sample from the transplant recipient (those with chromosomal integration will have high levels of HHV-6 DNA in the pretransplant sample).

[f]Systemic HHV-8 infection may be associated with body cavity lymphoma or multicentric Castleman disease. CSF, cerebrospinal fluid; FA, fluorescent antibody; Ig, immunoglobulin; NAAT, nucleic acid amplification test.

[g]Ward KN, Turner DJ, Parada XC, et al. Use of immunoglobulin G antibody avidity for differentiation of primary human herpesvirus 6 and 7 infections. *J Clin Microbiol*. 2001;39:959-963.

e-TABLE 16.5 Laboratory diagnosis of viral respiratory and gastrointestinal tract infections

Virus	Test Method	Comments
Respiratory tract infections		
Influenza A, B	NAAT	Most sensitive test
	FA stain	Less sensitive than NAAT
	Culture	Less sensitive than NAAT, requires viable virus
	Antigen detection	Low sensitivity, especially for pandemic 2009 H1N1 virus
Respiratory syncytial	NAAT	Most sensitive test
	FA stain	Less sensitive than NAAT
	Culture	Requires viable virus
	Rapid tests	Less sensitive than other methods
Parainfluenza	NAAT	Most sensitive test
	FA stain	Less sensitive than NAAT
	Culture	Requires viable virus
Human metapneumovirus	NAAT	Most sensitive test
	FA stain	Less sensitive than NAAT
Rhinovirus	NAAT	Most sensitive test; primers and probes must be selected carefully to detect all serotypes/genotypes. May be difficult or impossible to distinguish from enteroviruses
	Culture	Requires viable virus; is less sensitive than NAAT. Clade C rhinoviruses have not been successfully cultured as of time of writing
Coronaviruses (OC43, 229E, NL63, HKU1)	NAAT	Most sensitive test
SARS-CoV-2	NAAT	Most sensitive test
	Antigen	Less sensitive than NAAT, ability to perform in home
Adenoviruses	NAAT	Most sensitive test
	FA stain	Less sensitive than NAAT or culture
	FA	Less sensitive than culture
Gastrointestinal tract infections		
Rotavirus	NAAT	Most sensitive test
	Antigen detection	Less sensitive than NAAT
Norovirus	NAAT	Most sensitive test
	ELISA	Less sensitive than NAAT
Adenoviruses (group F, serotypes 40 and 41)	ELISA NAAT	Specific assay for serotypes 40 and 41 Most sensitive test
Astroviruses	NAAT	Most sensitive test

ELISA, enzyme-linked immunosorbent assay; FA, fluorescent antibody; NAAT, nucleic acid amplification test.

e-TABLE 16.6 Laboratory diagnosis of central nervous system infections

Syndrome	Virus	Method[a]
Meningitis	Enterovirus	NAAT
	Herpes simplex virus	NAAT
	Varicella-zoster virus	NAAT
	Human immunodeficiency virus	NAAT on plasma
Encephalitis in nonimmunosuppressed individuals	Herpes simplex West Nile virus and other arboviruses Varicella-zoster virus Epstein-Barr virus HHV-6 Rabies Mumps Lymphocytic choriomeningitis virus	NAAT IgM antibody assay on serum and CSF[b] NAAT, intrathecal antibody synthesis[c] NAAT, IgM antibody assay on serum and plasma NAAT NAAT on CSF and saliva, rabies antibody assay on CSF and serum,[d] FA stain of skin biopsy from nape of neck NAAT on CSF and saliva, IgM antibody assay on serum IgG and IgM antibody assays on CSF and serum (acute and convalescent samples), NAAT, culture
Encephalitis in immunosuppressed individuals	Cytomegalovirus Epstein-Barr virus Varicella-zoster virus JC virus	NAAT NAAT NAAT, intrathecal antibody synthesis[c] NAAT

[a]Indicated test is performed on CSF unless otherwise indicated.

[b]Testing should be performed on CSF and serum. Testing of CSF is more specific for current infection.

[c]Detection of intrathecal antibody synthesis has been shown to be more sensitive than NAAT for the diagnosis of varicella-zoster virus central nervous system vasculopathy. (Nagel MA, Cohrs RJ, Mahalingam R, et al. The varicella zoster virus vasculopathies: clinical, CSF, imaging, and virologic features. *Neurology*. 2008;70:853-860).

[d]CSF is preferred if the individual being evaluated has previously received rabies immunization. CSF, cerebrospinal fluid; HHV-6, human herpesvirus type 6; Ig, immunoglobulin; NAAT, nucleic acid amplification test.

e-TABLE 16.7 Laboratory diagnosis of congenital and neonatal viral infections

Virus	Recommended Testing
Cytomegalovirus	NAAT or culture of urine and/or saliva during first 2 weeks of life
Herpes simplex	NAAT or culture of a vesicle, CSF, blood (plasma, serum, or whole blood). Conjunctiva, nasopharynx, oropharynx, urine, and rectum/stool may also be tested
Human immunodeficiency virus (HIV)	NAAT for HIV RNA on plasma or DNA on whole blood
Rubella	IgM antibody assay of serum or plasma, NAAT or culture of urine, oropharynx, blood, stool, CSF. Amniotic fluid and products of conception can also be tested
Parvovirus B19	IgM antibody assay of serum or plasma, NAAT of plasma or serum
Hepatitis B	Hepatitis B surface antigen test on serum or plasma
Varicella-zoster virus	NAAT, culture, or FA stain on vesicle, CSF, or blood. NAAT on amniotic fluid
Enteroviruses	NAAT on plasma or serum, CSF, nasopharyngeal or oropharyngeal swab, stool, urine
Hepatitis C	NAAT on plasma or serum on two occasions between 2 and 6 months of age or HCV antibody assay at age ≥15 months of age

CSF, cerebrospinal fluid; FA, fluorescent antibody; Ig, immunoglobulin; NAAT, nucleic acid amplification assay.

e-TABLE 16.8 Laboratory diagnosis of human immunodeficiency and other retrovirus infections

Indication	Test
Routine diagnosis	ELISA for HIV antibodies[a]
Confirmation of a positive ELISA	Samples that are reactive on the screening HIV antibody or combined HIV antigen-antibody assay should be tested with a confirmatory antibody test that differentiates HIV-1 and HIV-2 antibodies.[b] Patients who are positive with this assay should have a second sample tested for HIV RNA. Rare patients who are reactive on the screening test and the confirmatory HIV-1/HIV-2 antibody assay but negative for HIV RNA should have additional testing performed under the direction of an HIV specialist. This testing might include a repeat of the screening assay, an HIV western blot, or an HIV DNA PCR assay. Patients who are negative with the confirmatory HIV-1/HIV-2 antibody assay should have an HIV RNA assay performed. Patients who are reactive on the screening assay, negative on the confirmatory HIV-1/HIV-2 antibody assay, and positive for HIV RNA are presumed to have recent infection and should be referred immediately to an HIV specialist[c]
Blood donor screening	ELISA for antibodies to HIV-1 and -2, HIV RNA assay
Infant born to infected mother	HIV RNA or DNA assay at 14-21 days of age, 1-2 months, and 4-6 months[d]
Acute antiretroviral syndrome	HIV RNA assay
Prognosis	Quantitative HIV RNA assay
Response to therapy	Quantitative HIV RNA assay
Resistance to antiretroviral drugs	Genotypic assay based on nucleotide sequencing of the relevant genes, or phenotypic assay based on inserting relevant genes from the patient's HIV strain into a laboratory vector
Diagnosis of HIV-2 infection	ELISA for HIV-2 antibodies[e]
Monitoring of HIV-2 infection	HIV-2 RNA assay[f]
Diagnosis of HTLV-1 or -2 infection	HTLV-1 and 2 antibody ELISA assays
Confirmation of HTLV-1 or -2 infection	HTLV-1 and -2 western blot, line immunoassay, or immunofluorescent antibody assay[e]

[a]Most FDA licensed tests detect antibodies to both HIV-1 and -2. Third-generation antibody assays are preferred because they detect immunoglobulin M (IgM) as well as IgG antibodies and thus become positive earlier after infection than assays that detect only IgG antibodies. An alternative approach is to use a fourth-generation test that measures HIV p24 antigen in addition to HIV antibodies.

[b]A licensed rapid HIV antibody test approved by the Food and Drug Administration (FDA) is now available that differentiates HIV-1 and HIV-2 antibodies.

[c]Branson BM, Mermin J. Establishing the diagnosis of HIV infection: new tests and a new algorithm for the United States. *J Clin Virol*. 2011;52(Suppl 1):S3-S4.

[d]DNA PCR may be preferred while the infant is on prophylactic antiretroviral treatment because of the possibility that antiretroviral therapy may make HIV RNA undetectable.

[e]Most HIV antibody assays currently in use detect antibodies to both HIV-1 and HIV-2, but only one FDA-licensed test differentiates between HIV-1 and HIV-2 antibodies.

[f]Available only in selected reference laboratories. ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; HTLV, human T-cell lymphoma or leukemia virus; PCR, polymerase chain reaction.

e-TABLE 16.9 Laboratory diagnosis of tropical and other geographically localized viral infections

Virus or Syndrome	Location	Preferred Tests
Sin Nombre virus	Western United States	Virus-specific IgM antibody assay, NAAT
Other hantaviruses	Asia, Europe	Virus-specific IgM antibody assay, NAAT
Colorado tick fever	Western United States	Virus-specific IgM antibody assay, NAAT
Dengue fever	Asia, Central and South America	Virus-specific IgM antibody assay, NAAT
Filoviruses (Ebola and Marburg viruses)	Central Africa	Virus-specific IgM antibody assay, NAAT
Arenaviruses (eg, Lassa, Junin, many others)	West Africa (Lassa fever), South American (Junin)	Virus-specific IgM antibody assay, NAAT, culture
Hendra and Nipah viruses	Australia, Malaysia, Bangladesh, India	Virus-specific IgM antibody assay, NAAT
Yellow fever	Central Africa, Amazon region	Virus-specific IgM antibody assay, NAAT
Crimean-Congo hemorrhagic fever and other bunyavirus infections	Asia, Africa, Europe	Virus-specific IgM antibody assay, antigen-capture ELISA, culture, NAAT
Rift Valley fever	East Africa	Virus-specific IgM antibody assay, antigen-capture ELISA, culture, NAAT, culture
Chikungunya and other alphavirus infections (O'nyong nyong, sindbis, Mayaro, Ross River)	Africa, India, Central and South America, Europe, Australia (Ross River)	Virus-specific IgM antibody assay, culture, NAAT

ELISA, enzyme-linked immunosorbent assay; IgM, immunoglobulin M; NAAT, nucleic acid amplification test.

Virus Discovery and Metagenomics

Experimental methodologies used for viral discovery have traditionally relied on classic approaches such as electron microscopy, cell culture, and antibody-based detection. Despite the success of these approaches, which have resulted in the discovery of many viruses throughout the years, each of these methods suffers from significant limitations. For example, electron microscopy requires high viral titers and may not provide the necessary resolution to unambiguously identify the type of virus even when viral particles are observed. Many viruses cannot be cultured, and those that can often require very specific cell types; crucially, there is no universal cell line capable of broadly supporting growth of all, or even a majority of, known viruses. Antibody-based methods are limited in breadth, require the development of specific reagents for a given viral group, and, most critically, require selection of specific candidates based on prior knowledge in order to be useful in identifying a virus. Due to these limitations, there have been ongoing efforts to develop additional complementary strategies for detecting and discovering novel viruses. In the last decade of the 20th century, various molecular methods were developed in efforts to circumvent limitations of these classic approaches. These molecular methods, including library immunoscreening, degenerate PCR, and representational difference analysis, led to the discovery of many clinically important viruses including HCV, Kaposi sarcoma herpesvirus (KSHV), and others.[41]

The 21st century has witnessed a revolution in the field of virus discovery as evidenced by the dramatic increase in the rate of identification of novel viruses. This increase has been driven largely by the advent of the genomic era, which has created novel sequencing technologies that in turn have geometrically increased the volume of sequencing data that can be generated. The combination of increased sequences in public databases and increased sequencing capacity has enabled the development of new, sophisticated sequence-based approaches for viral detection and discovery. Even existing methods, such as consensus PCR approaches, have benefited from the geometric growth in sequence databases. With the availability of many more viral genome sequences, improved consensus PCR primers can be designed for many viral taxa.

All of the recently developed viral discovery methods are based on detection of viral nucleic acids. Fundamentally, the major challenge in sequence-based viral discovery is identifying the relatively few copies of viral DNA or RNA in specimens that typically contain a vast excess of host genomic DNA and/or RNA. In theory, given unlimited sequencing capacity, there would be no need to be concerned with the host; exhaustive direct sequencing of a given sample should reveal the presence of any viral sequences in the sample no matter how low the abundance of the virus. However, practical limitations on sequencing, either in terms of sheer capacity or cost, have until recently required that additional methods be employed to effectively increase the relative percentage of viral sequence to host sequence. For example, propagation of a virus in cell culture is one means of increasing the relative abundance of viral sequences. Physical purification of virions, by ultracentrifugation or nuclease treatment to eliminate nonencapsidated nucleic acids, is another means of enriching for viral sequences. Subtractive hybridization strategies achieve a similar end point by physically reducing the amount of host sequences. However, we are rapidly approaching a point where sequencing capacity and costs are no longer limiting.

One potential limitation in sequence-based virus discovery is the quantity of nucleic acids available for sequencing. To circumvent this challenge, multiple experimental approaches to increase the total abundance of nucleic acids to levels amenable to further analysis have also been developed. An essential feature of all of these amplification strategies is that no prior knowledge regarding the nature of the sequences to

be amplified is required because it is unknown what type of virus may be present. Several different approaches for enriching for viral nucleic acids for viral discovery as well as library preparation strategies are illustrated in Figure 16.7.

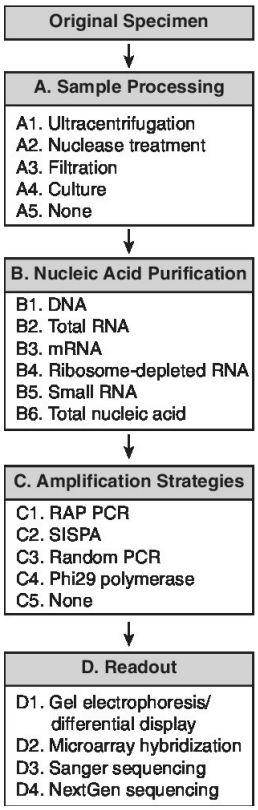


FIGURE 16.7 **Pathogen discovery methods**. Recently developed sequence-based pathogen discovery methods all fundamentally follow a similar overall scheme. A clinical specimen of interest is collected. **A**. If desired, samples can be manipulated to increase the ratio of virus to host by various methods. **B**. Nucleic acid species of interest is isolated. **C**. In cases where the quantity of purified nucleic acid is limiting, random sequencing-independent amplification is applied. **D**. A readout method is selected. For example, the identification of human bocavirus used the sequence of steps A1-A2-B1-C3-D3. The identification of Merkel polyomavirus used the sequence A5-B3-C5-D4.

For sequence-based detection technologies, a key step is the computational analysis required to identify a sequence as viral in nature. In early efforts, only a few sequences were generated and thus analysis could easily be done via commonly used web-interface software. With advances in sequencing capacity, the number of sequences generated from a sample increased initially to hundreds or thousands and now can easily surpass a million sequences or more. In order to efficiently analyze this volume of sequence data, automated high-throughput bioinformatics pipelines had to be developed. Although many variations on this theme exist, most computational pipelines utilize very similar overall strategies. Typically, sequence alignment software, such as BLAST, is used to compare the sequences generated from a sample to established public databases. Sequences with perfect or near-perfect alignments to known sequences (host, viral, bacterial, etc.) can then be categorized based on the identity of the reference sequence to which a given read aligned. Sequences derived from novel viruses are those that have only limited similarity to known viruses in the available databases. For more definitive characterization of the relatedness of these sequences to known viruses, phylogenetic methods are subsequently used.

As mentioned previously, a key driving factor in the advances in virus discovery has been the evolution of sequencing technology. For over 30 years, from its inception in the late 1970s until the late 2000s, Sanger dideoxy sequencing was the dominant sequencing technology in use, and all efforts to identify viruses using sequencing-based strategies relied on this technology. At its highest throughput, automated 96-capillary Sanger sequencing could generate up to ~100 000 bp of sequence in a single run. Starting in 2005, the first of the so-called next-generation sequencing (NGS) platforms was introduced.[49] These platforms dramatically increased the quantity of sequence that could be produced. For example, the Roche/454 platform in its initial format was capable of generating 100 000 reads of 100 bp length in a single run, or ~100-fold greater sequence than a run on the previous instrument standard, the ABI 3730. Over the next few years, this platform rapidly evolved such that by 2009, the Roche/454 platform could readily generate 1 000 000 reads that averaged 400 bp each, or 400 megabases (MB) of total sequence. Two other major NGS platforms, Solexa (Illumina) and SOLiD (ABI), also emerged during this time (reviewed in[48]), with Illumina the dominant platform for more than a decade. At the current time, top-line sequencers are capable of generating billions of reads in less than a day and terabases of sequence in a single run. It is the unprecedented growth in sequencing capacity offered by these new platforms that has transformed the process of virus discovery. In addition, metagenomic NGS is now available clinically for broad-based pathogen detection in plasma, CSF, and bronchial alveolar lavage fluids.[4,51]

Representative Novel Human Viruses Discovered

A number of new human viruses were discovered from 2000 to 2010 using these more unbiased molecular approaches. These include parvovirus 4, anelloviruses, bocaviruses, multiple polyomaviruses, astroviruses, rhinoviruses and other picornaviruses, and arenaviruses.[1,2,5,18,22,29,35,37,39,40,54,57,71,72,81,82] Among the most important discoveries were the surprisingly common rhinovirus C lineage and several novel human astroviruses associated with meningoencephalitis. More recently, these techniques in combination with cell culture

have also been critical for finding tick-borne illnesses such as severe fever and thrombocytopenia virus,[87] Heartland,[50] and Bourbon virus,[43] novel zoonotic respiratory pathogens such as MERS-CoV,[80] variegated squirrel bornavirus, and SARS-CoV-2. In addition to the minority of novel viruses that have clearly been associated with pathogenicity in humans, many newly identified human, animal, and arthropod viruses await further characterization and assessment of their zoonotic potential and/or clinical relevance.[27]

Prospects and Challenges for the Future

Advances in molecular methods have dramatically increased the rate at which viral genomes can be detected, resulting in our awareness of the existence of many more viral genomes than just a few years ago. For example, following the initial discoveries of BK and JC viruses by culture in 1971, there were no additional polyomaviruses of humans identified until 2007. Since then, a total of 12 new polyomaviruses have been identified in humans, all by molecular methods. Parallel situations exist in many viral taxa, wherein geometric increases in the number of known members have been observed. With the continued growth in sequencing capacity, there is little doubt that many more viruses will be identified in the upcoming years. Recently, specific efforts to comprehensively catalog the spectrum of viruses present in humans have begun. One objective of the international Human Microbiome Project is to define the set of viruses present in different anatomic sites in a set of "healthy" adults. These and other similar efforts to define the "viromes" of humans, animals, plants, or environmental niches will undoubtedly uncover a tremendous diversity of new viral genomes. While our ability to identify viral nucleic acids and genomes has grown at an astounding rate, our ability to define the molecular properties and the biological relevance of these new agents has unfortunately lagged behind. In order to effectively study these new viruses, commensurate new developments in traditional virology must occur. For example, novel methods of virus culture must be developed in order to keep pace with the rate of discovery of new viral sequences, as the ability to propagate a virus in culture is, in many instances, the rate-limiting step in our ability to further characterize a virus and to define its pathogenicity. Many of the viruses being discovered today by sequencing-based molecular methods are unlikely to grow in the traditional cell lines utilized by virologists. As an example, the advent of differentiated primary respiratory epithelial cultures has opened new frontiers by enabling the culture of several newly identified respiratory viruses, such as human bocavirus[15] and coronavirus HKU,[62] that have failed to grow previously in traditional respiratory tract–derived cell lines. Two- and three-dimensional organoids composed of multiple cell types that more closely mimic the complexity of tissues provide another avenue for cultivation of viruses. Another area of great importance is the development of robust animal models, including those with impaired innate immunity and/or engrafted with human cells, for these new agents, which will be a critical part of efforts to define pathogenicity and disease causality. Finally, the criteria used for defining "pathogenicity" must also be revisited. While Koch's postulates, the 100-plus-year-old gold standard for proof of disease causation, have served us well in the past, this era of molecular virus discovery necessitates an enhanced emphasis on the role of epidemiologic and seroepidemiologic approaches in defining disease associations.[19] Furthermore, more nuanced conceptions of microbially induced disease, such as disease as a consequence of imbalance or perturbation of host microbial populations, must also be considered. In summary, technology-driven advances in sequencing have led to a golden era of discovery of new viruses in the first decade of the 21st century. Hopefully, parallel advances in understanding the biology of these new agents will come to fruition in the next decades of the 21st century.

Perspective

Diagnostic virology is at a critical point in its history, especially in the wake of the COVID-19 pandemic. The field has matured considerably, with detection of a selected group of viral pathogens emerging as an important component of clinical laboratory services at major medical centers. Molecular detection of respiratory viruses; diagnosis and monitoring of viral infections, especially CMV, EBV, and BK virus in immunocompromised patients; viral load and antiviral susceptibility testing for HIV, HCV, and HBV; and testing for HPV have all become mainstream diagnostic procedures. After consolidating the conversion to molecular methods, the methodology of diagnostic virology is again evolving rapidly, with newly available distributed CLIA-waived antigen and molecular testing at home and high complexity metagenomic NGS in specialized laboratories. The development of standardized commercial molecular tests that have passed through the review processes of the FDA continues to gain momentum, while reimbursement for multiplex testing remains a challenge. Greater availability of therapeutics for viral diseases through monoclonal antibodies and small molecule antivirals will help drive continued innovation and demand for rapid and widespread viral diagnostics. While new innovations in molecular methods from CRISPR-Cas detection systems to digital PCR and NGS show promise, it is the front-end automation and dispersion of more mature diagnostic methods such as qPCR and antigen testing that has had the greatest impact over the past half decade. In addition, there is little doubt that unforeseen technological advances will drive the evolution of the field in directions that are currently impossible to predict.

Disclosures

Dr. Storch serves on the medical advisory board of Roche Molecular Systems and has served as a consultant to Idaho Technology, PrimeraDx, Abbott, and Diagnostic Hybrids. He has received an honorarium from GenProbe and Meridian. He is currently performing research funded by Luminex and Meridian. Dr. Greninger reports contract testing from Abbott, Cepheid, Novavax, Pfizer, Janssen, and Hologic and research support from Gilead and Merck, outside of the described work. Dr. Wang and Dr. Jerome have no disclosures.

REFERENCES

1. Allander T, Andreasson K, Gupta S, et al. Identification of a third human polyomavirus. J Virol. 2007;81(8):4130-4136.

2. Arthur JL, Higgins GD, Davidson GP, Givney RC, Ratcliff RM. A novel bocavirus associated with acute gastroenteritis in Australian children. PLoS Pathog. 2009;5(4):e1000391.

3. Bedson S, Bland J. Complement-fixation with filterable viruses and their antisera. Br J Exp Pathol. 1929 (1929):393-404.

4. Blauwkamp TA, Thair S, Rosen MJ, et al. Analytical and clinical validation of a microbial cell-free DNA sequencing test for infectious disease. Nat Microbiol. 2019;4(4):663-674.

5. Briese T, Paweska JT, McMullan LK, et al. Genetic detection and characterization of Lujo virus, a new hemorrhagic fever-associated arenavirus from southern Africa. PLoS Pathog. 2009;5(5):e1000455.

6. Bright RA, Medina M, Xu X, et al. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to

2005: a cause for concern. Lancet. 2005;366(9492):1175-1181.

7. Broughton JP, Deng X, Yu G, et al. CRISPR-Cas12-based detection of SARS-CoV-2. Nat Biotechnol. 2020;38(7):870-874.

8. Buxton RC, Edwards B, Juo RR, et al. Development of a sensitive chemiluminescent neuraminidase assay for the determination of influenza virus susceptibility to zanamivir. Anal Biochem. 2000;280(2):291-300.

9. Chapman NM, Tracy S, Gauntt CJ, Fortmueller U. Molecular detection and identification of enteroviruses using enzymatic amplification and nucleic acid hybridization. J Clin Microbiol. 1990;28(5):843-850.

10. Cheval J, Sauvage V, Frangeul L, et al. Evaluation of high-throughput sequencing for identifying known and unknown viruses in biological samples. J Clin Microbiol. 2011;49(9):3268-3275.

11. Chou S. Cytomegalovirus UL97 mutations in the era of ganciclovir and maribavir. Rev Med Virol. 2008;18(4):233-246.

12. Dalmat RR, Makhsous N, Pepper GG, et al. Limited marginal utility of deep sequencing for HIV drug resistance testing in the age of integrase inhibitors. J Clin Microbiol. 2018;56(12):e01443-18.

13. de Vries M, Deijs M, Canuti M, et al. A sensitive assay for virus discovery in respiratory clinical samples. PLoS One. 2011;6(1):e16118.

14. Deiman B, van Aarle P, Sillekens P. Characteristics and applications of nucleic acid sequence-based amplification (NASBA). Mol Biotechnol. 2002;20(2):163-179.

15. Dijkman R, Koekkoek SM, Molenkamp R, Schildgen O, van der Hoek L. Human bocavirus can be cultured in differentiated human airway epithelial cells. J Virol. 2009;83(15):7739-7748.

16. Eggers M, Bäder U, Enders G. Combination of microneutralization and avidity assays: improved diagnosis of recent primary human cytomegalovirus infection in single serum sample of second trimester pregnancy. J Med Virol. 2000;60(3):324-330.

17. Eisen HN, Siskind GW. Variations in affinities of antibodies during the immune response. Biochemistry. 1964;3:996-1008.

18. Finkbeiner SR, Allred AF, Tarr PI, et al. Metagenomic analysis of human diarrhea: viral detection and discovery. PLoS Pathog. 2008;4(2):e1000011.

19. Fredricks DN, Relman DA. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. Clin Microbiol Rev. 1996;9(1):18-33.

20. Gallo D, George JR, Fitchen JH, Goldstein AS, Hindahl MS. Evaluation of a system using oral mucosal transudate for HIV-1 antibody screening and confirmatory testing. OraSure HIV Clinical Trials Group. JAMA. 1997;277(3):254-258.

21. Gaudreau A, Hill E, Balfour HH, Erice A, Boivin G. Phenotypic and genotypic characterization of acyclovir-resistant herpes simplex viruses from immunocompromised patients. J Infect Dis. 1998;178(2):297-303.

22. Gaynor AM, Nissen MD, Whiley DM, et al. Identification of a novel polyomavirus from patients with acute respiratory tract infections. PLoS Pathog. 2007;3(5):e64.

23. Giachetti C, Linnen JM, Kolk DP, et al. Highly sensitive multiplex assay for detection of human immunodeficiency virus type 1 and hepatitis C virus RNA. J Clin Microbiol. 2002;40(7):2408-2419.

24. Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. Genome Res. 1996;6(10):995-1001.

25. Ginocchio CC, Swierkosz E, McAdam AJ, et al. Multicenter study of clinical performance of the 3M Rapid Detection RSV test. J Clin Microbiol. 2010;48(7):2337-2343.

26. Grant PM, Zolopa AR. The use of resistance testing in the management of HIV-1-infected patients. Curr Opin HIV AIDS. 2009;4(6):474-480.

27. Greninger AL. A decade of RNA virus metagenomics is (not) enough. Virus Res. 2018;244:218-229.

28. Greninger AL, Jerome KR. The first quarter of SARS-CoV-2 testing: the University of Washington Medicine Experience. J Clin Microbiol. 2020;58(8). Accessed June 12, 2021. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7383557/

29. Greninger AL, Runckel C, Chiu CY, et al. The complete genome of klassevirus—a novel picornavirus in pediatric stool. Virol J. 2009;6:82.

30. Greninger AL, Zerr DM, Qin X, et al. Rapid metagenomic next-generation sequencing during an investigation of hospital-acquired human parainfluenza virus 3 infections. J Clin Microbiol. 2017;55(1):177-182.

31. Gresham D, Curry B, Ward A, et al. Optimized detection of sequence variation in heterozygous genomes using DNA microarrays with isothermal-melting probes. Proc Natl Acad Sci U S A. 2010;107(4):1482-1487.

32. Guatelli JC, Whitfield KM, Kwoh DY, et al. Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. Proc Natl Acad Sci U S A. 1990;87(5):1874-1878.

33. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res. 1996;6(10):986-994.

34. Higuchi R, Dollinger G, Walsh PS, Griffith R. Simultaneous amplification and detection of specific DNA sequences. Biotechnology (N Y). 1992;10(4):413-417.

35. Holtz LR, Finkbeiner SR, Zhao G, et al. Klassevirus 1, a previously undescribed member of the family Picornaviridae, is globally widespread. Virol J. 2009;6:86.

36. Jones MS, Kapoor A, Lukashov VV, et al. New DNA viruses identified in patients with acute viral infection syndrome. J Virol. 2005;79(13):8230-8236.

37. Jones MS, Lukashov VV, Ganac RD, Schnurr DP. Discovery of a novel human picornavirus in a stool sample from a pediatric patient presenting with fever of unknown origin. J Clin Microbiol. 2007;45(7):2144-2150.

38. Joung J, Ladha A, Saito M, et al. Detection of SARS-CoV-2 with SHERLOCK One-Pot Testing. N Engl J Med. 2020;383(15):1492-1494.

39. Kapoor A, Victoria J, Simmonds P, et al. A highly prevalent and genetically diversified Picornaviridae genus in South Asian children. Proc Natl Acad Sci U S A. 2008;105(51):20482-20487.

40. Kapoor A, Slikas E, Simmonds P, et al. A newly identified bocavirus species in human stool. J Infect Dis. 2009;199(2):196-200.

41. Kellam P. Molecular identification of novel viruses. Trends Microbiol. 1998;6(4):160-165.

42. Kohler P, Tijet N, Kim HC, et al. Dissemination of Verona Integron-encoded Metallo-β-lactamase among clinical and environmental Enterobacteriaceae isolates in Ontario, Canada. Sci Rep. 2020;10(1):18580.

43. Kosoy OI, Lambert AJ, Hawkinson DJ, et al. Novel thogotovirus associated with febrile illness and death, United States, 2014. Emerg Infect Dis. 2015;21(5):760-764.

44. Kozal MJ, Shah N, Shen N, et al. Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays. Nat Med. 1996;2(7):753-759.

45. Liu C. Rapid diagnosis of human influenza infection from nasal smears by means of fluorescein-labeled antibody. Proc Soc Exp Biol Med. 1956;92(4):883-887.

46. Lurain NS, Chou S. Antiviral drug resistance of human cytomegalovirus. Clin Microbiol Rev. 2010;23(4):689-712.

47. Mahony J, Chong S, Merante F, et al. Development of a respiratory virus panel test for detection of twenty human respiratory viruses by use of multiplex PCR and a fluid microbead-based assay. J Clin Microbiol. 2007;45(9):2965-2970.

48. Mardis ER. A decade's perspective on DNA sequencing technology. Nature. 2011;470(7333):198-203.

49. Margulies M, Egholm M, Altman WE, et al. Genome sequencing in microfabricated high-density picolitre reactors. Nature. 2005;437(7057):376-380.

50. McMullan LK, Folk SM, Kelly AJ, et al. A new phlebovirus associated with severe febrile illness in Missouri. N Engl J Med. 2012;367(9):834-841.

51. Miller S, Naccache SN, Samayoa E, et al. Laboratory validation of a clinical metagenomic sequencing assay for pathogen detection in cerebrospinal fluid. Genome Res. 2019;29(5):831-842.

52. Nagel MA, Cohrs RJ, Mahalingam R, et al. The varicella zoster virus vasculopathies: clinical, CSF, imaging, and virologic features. Neurology. 2008;70(11):853-860.

53. Nguyen HT, Sheu TG, Mishin VP, Klimov AI, Gubareva LV. Assessment of pandemic and seasonal influenza A (H1N1) virus susceptibility to neuraminidase inhibitors in three enzyme activity inhibition assays. Antimicrob Agents Chemother. 2010;54(9):3671-3677.

54. Nishizawa T, Okamoto H, Konishi K, et al. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem Biophys Res Commun. 1997;241(1):92-97.

55. Notomi T, Okayama H, Masubuchi H, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 2000;28(12):E63.

56. Ou JH, Laub O, Rutter WJ. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. Proc Natl Acad Sci U S A. 1986;83(6):1578-1582.

57. Palacios G, Druce J, Du L, et al. A new arenavirus in a cluster of fatal transplant-associated diseases. N Engl J Med. 2008;358(10):991-998.

58. Pandori MW, Hackett J, Louie B, et al. Assessment of the ability of a fourth-generation immunoassay for human immunodeficiency virus (HIV) antibody and p24 antigen to detect both acute and recent HIV infections in a high-risk setting. J Clin Microbiol. 2009;47(8):2639-2642.

59. Paredes MI, Lunn SM, Famulare M, et al. Associations between SARS-CoV-2 variants and risk of COVID-19 hospitalization among confirmed cases in Washington State: a retrospective cohort study. Clin Infect Dis. 2022;75(1):e536-e544.

60. Parkin NT, Lie YS, Hellmann N, et al. Phenotypic changes in drug susceptibility associated with failure of human immunodeficiency virus type 1 (HIV-1) triple combination therapy. J Infect Dis. 1999;180(3):865-870.

61. Perchetti GA, Huang M-L, Mills MG, Jerome KR, Greninger AL. Analytical Sensitivity of the Abbott BinaxNOW COVID-19 Ag Card. J Clin Microbiol. 2021;59(3):e02880-20.

62. Pyrc K, Sims AC, Dijkman R, et al. Culturing the unculturable: human coronavirus HKU1 infects, replicates, and produces progeny virions in human ciliated airway epithelial cell cultures. J Virol. 2010;84(21):11255-11263.

63. Rand KH, Rampersaud H, Houck HJ. Comparison of two multiplex methods for detection of respiratory viruses: FilmArray RP and xTAG

RVP. J Clin Microbiol. 2011;49(7):2449-2453.

64. Ray CG, Minnich LL. Efficiency of immunofluorescence for rapid detection of common respiratory viruses. J Clin Microbiol. 1987;25(2):355-357.

65. Reiber H, Lange P. Quantification of virus-specific antibodies in cerebrospinal fluid and serum: sensitive and specific detection of antibody synthesis in brain. Clin Chem. 1991;37(7):1153-1160.

66. Rotbart HA. Enzymatic RNA amplification of the enteroviruses. J Clin Microbiol. 1990;28(3):438-442.

67. Rousseau S, Hedman K. Rubella infection and reinfection distinguished by avidity of IgG. Lancet. 1988;1(8594):1108-1109.

68. Safrin S, Elbeik T, Phan L, et al. Correlation between response to acyclovir and foscarnet therapy and in vitro susceptibility result for isolates of herpes simplex virus from human immunodeficiency virus-infected patients. Antimicrob Agents Chemother. 1994;38: 1246-1250.

69. Saiki RK, Scharf S, Faloona F, et al. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 1985;230(4732):1350-1354.

70. Schlaberg R, Ampofo K, Tardif KD, et al. Human bocavirus capsid messenger RNA detection in children with pneumonia. J Infect Dis. 2017;216(6):688-696.

71. Schowalter RM, Pastrana DV, Pumphrey KA, Moyer AL, Buck CB. Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin. Cell Host Microbe. 2010;7(6):509-515.

72. Scuda N, Hofmann J, Calvignac-Spencer S, et al. A novel human polyomavirus closely related to the african green monkey-derived lymphotropic polyomavirus. J Virol. 2011;85(9):4586-4590.

73. Simen BB, Simons JF, Hullsiek KH, et al. Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes. J Infect Dis. 2009;199(5):693-701.

74. Stránská R, Schuurman R, Scholl DR, et al. ELVIRA HSV, a yield reduction assay for rapid herpes simplex virus susceptibility testing. Antimicrob Agents Chemother. 2004;48(6): 2331-2333.

75. Suligoi B, Galli C, Massi M, et al. Precision and accuracy of a procedure for detecting recent human immunodeficiency virus infections by calculating the antibody avidity index by an automated immunoassay-based method. J Clin Microbiol. 2002;40(11):4015-4020.

76. Svraka S, Rosario K, Duizer E, et al. Metagenomic sequencing for virus identification in a public-health setting. J Gen Virol. 2010;91(Pt 11):2846-2856.

77. Tebas P, Stabell EC, Olivo PD. Antiviral susceptibility testing with a cell line which expresses beta-galactosidase after infection with herpes simplex virus. Antimicrob Agents Chemother. 1995;39(6):1287-1291.

78. The TH, van der Ploeg M, van den Berg AP, et al. Direct detection of cytomegalovirus in peripheral blood leukocytes—a review of the antigenemia assay and polymerase chain reaction. Transplantation. 1992;54(2):193-198.

79. Urnovitz HB, Sturge JC, Gottfried TD. Increased sensitivity of HIV-1 antibody detection. Nat Med. 1997;3(11):1258.

80. van Boheemen S, de Graaf M, Lauber C, et al. Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. mBio. 2012;3(6):e00473-12.

81. van der Meijden E, Janssens RWA, Lauber C, et al. Discovery of a new human polyomavirus associated with trichodysplasia spinulosa in an immunocompromized patient. PLoS Pathog. 2010;6(7):e1001024.

82. van Leeuwen M, Williams MMW, Koraka P, et al. Human picobirnaviruses identified by molecular screening of diarrhea samples. J Clin Microbiol. 2010;48(5):1787-1794.

83. Wang D, Coscoy L, Zylberberg M, et al. Microarray-based detection and genotyping of viral pathogens. Proc Natl Acad Sci U S A. 2002;99(24):15687-15692.

84. Weller TH, Enders JF. Production of hemagglutinin by mumps and influenza A viruses in suspended cell tissue cultures. Proc Soc Exp Biol Med. 1948;69(1):124-128.

85. Whitcomb JM, Huang W, Fransen S, et al. Development and characterization of a novel single-cycle recombinant-virus assay to determine human immunodeficiency virus type 1 coreceptor tropism. Antimicrob Agents Chemother. 2007;51(2):566-575.

86. Yang J, Yang F, Ren L, et al. Unbiased parallel detection of viral pathogens in clinical samples by use of a metagenomic approach. J Clin Microbiol. 2011;49(10):3463-3469.

87. Yu X-J, Liang M-F, Zhang S-Y, et al. Fever with thrombocytopenia associated with a novel bunyavirus in China. N Engl J Med. 2011;364(16):1523-1532.

CHAPTER 17

Giant Viruses

Jônatas Santos Abrahão

Introduction and history

Classification and evolutionary relationships

Phycodnaviridae

Virion structure

Genome structure and organization

Stages of replication

Mimiviridae

Virion structure

Genome structure and organization

Stages of replication

Virophages

Marseilleviridae

Virion structure

Genome structure and organization

Stages of replication

Pandoraviruses

Virion structure

Genome structure and organization

Stages of replication

"Extended *Asfarviridae*": faustovirus, kaumoebavirus, and pacmanvirus

Virion structure

Genome structure and organization

Stages of replication

Pithovirus, cedratvirus, and orpheovirus

Virion structure

Genome structure and organization

Stages of replication

Mollivirus

Virion structure

Genome structure and organization

Stages of replication

Medusavirus

Virion structure

Genome structure and organization

Stages of replication

Perspectives

Acknowledgments

Introduction and History

The discovery and characterization of giant viruses in the last decades have raised fundamental questions regarding the boundaries of the virosphere. Not only are their virions remarkably complex and gigantic, but their double-stranded DNA (dsDNA) genomes are often in the megabase range and encode proteins that were previously thought to be exclusive of cellular organisms (Fig. 17.1).[1] The term "giant virus" is quite flexible and often used interchangeably with "large virus" to refer to the same virus.[10,51,99,144] In the context of unicellular eukaryote–infecting viruses, the term "giant virus" was first used in 1999, to designate Paramecium bursaria Chlorella virus, the defining member of a group of algae-infecting viruses whose particles are 140-190 nm in diameter.[172] However, the description of Acanthamoeba polyphaga mimivirus (APMV) in 2003, an amoeba-infecting virus whose virions are ~750 nm, popularized the use of the term.[82] Although some bacteriophages with large genomes and particles have been described ("jumbo phages"), the term giant virus has been used to refer to an evolutionary lineage of viruses belonging to the phylum *Nucleocytoviricota*, mostly those infecting unicellular eukaryotes, including amoebas, flagellates, algae, and others.[77,186] Therefore, in this chapter, we discuss the main features of this group. We even discuss some nucleocytoviruses of unicellular eukaryotes with relatively small capsids (~100 nm), because they highlight structural and evolutionary features observed in their giant relatives.

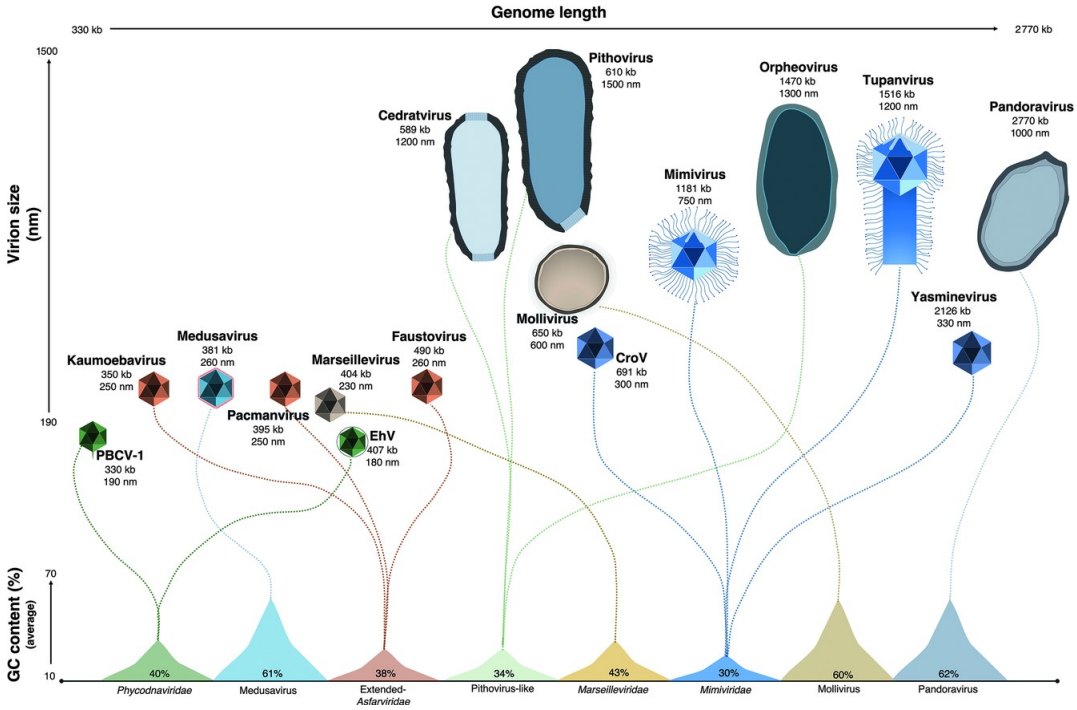


FIGURE 17.1 **Schematic illustration of genomic and structural features of giant virus representatives**. GC content and virion dimensions are shown on the y-axis. Genome length is plotted on the x-axis.

Seminal studies in the 1970s described the presence of viruslike particles (VLPs) with diameters of hundreds of nanometers in association with eukaryotic, mostly unicellular, organisms containing chloroplasts, including *Heteromastix* sp., *Oedogonium* sp., *Hymenomonas carterae*, and *Monodus* sp.[60,121,122] Some of these studies have advanced the chemical characterization of VLPs, such as those related to *Uronema gigas*, revealing particles around 400 nm, containing dsDNA and a guanine/cytosine (G/C) content of 73.5%.[60] Interestingly, about 10% of those purified VLPs had a tail measuring about 1 μm in length, which could be associated with adhesion of the virus to the algal wall and trigger the onset of infection.[46] However, due to technical difficulties related to the propagation of VLPs, most studies in the 1970s did not characterize these large VLPs further.

The propagation and characterization of large eukaryotic algae viruses started to be described systematically in the early 1980s. The so-called chloroviruses, currently belonging to the family *Phycodnaviridae*, have been described as infecting different species of the eukaryotic algal genus *Chlorella*.[97,173,174] Although these algae can be observed as free-living entities in nature, they usually live as symbionts with a variety of organisms, including the coelenterate *Hydra viridis*, the ciliate *Paramecium bursaria* and the heliozoan *Acanthocystis turfacea*. Several large DNA viruses such as Hydra viridis Chlorella virus, Paramecium bursaria Chlorella virus, and Acanthocystis turfacea Chlorella virus were isolated from these symbiotic species of *Chlorella*. Because *Chlorella* could be cultured in the laboratory, it was possible to establish innovative protocols for studying the newly discovered chloroviruses, such as an algal adaptation of the plaque assay.[168] Therefore, the description of the first chloroviruses represents a hallmark in the study of unicellular eukaryote–infecting giant viruses.

As the number of complete viral genomes in the databases increased, comprehensive analyses published in the early 2000s indicated that phycodnaviruses share a set of nine genes with viruses belonging to other dsDNA virus families, such as *Poxviridae*, *Iridoviridae*, and *Asfarviridae*. Twenty-two genes were shared by at least three of these families, which is a substantial number if we consider other distantly related viral families. Other shared features include particle sizes of a few hundred nanometers, genomes more than 200 kilobase pairs (kb) long, and a partially or completely cytoplasmic mode of replication. The term nucleocytoplasmic large DNA virus (NCLDV) was then proposed to designate this group with a hypothetically monophyletic origin.[72]

However, in 2003, the NCLDV group gained a notable member: mimivirus. The discovery of this virus dates back to 1992, when Dr. T.J. Rowbotham's team isolated an obligate intracellular parasite of amoeba from a cooling tower. This intracellular parasite stained Gram positive and was termed Bradfordcoccus. After unsuccessful attempts to identify it, a few years later, this supposed bacterial agent was sent to the Unité des Rickettsies in Marseille, France, and by electron microscopy, it was possible to observe, in the cytoplasm of amoebas, isometric capsids (500 nm) covered by long fibrils, giving the particles a total size of ~750 nm.[82] Together with these micrographs, genomic sequencing confirmed that Bradfordcoccus was in fact a gigantic virus with a ~1.2-Mb genome.[124] This new virus was named Acanthamoeba polyphaga mimivirus (mimivirus stands for *mi*micking *mi*crobe).

The discovery of mimivirus prompted further studies and the discovery of even larger viruses associated with protists.[3,12,87,119] New unicellular eukaryotic hosts have been explored, and the ubiquity of giant viruses has been revealed by comprehensive metagenomic studies in the most diverse biomes on the planet.[20,56,64,126,146] The origin of NCLDVs, now incorporated as members of the phylum *Nucleocytoviricota*, has been the subject of intense study and debate in recent years, highlighting exclusive biological characteristics of this remarkable group of viruses. Finally, the ecological role of giant viruses represents one of the frontiers of current knowledge and has revealed sophisticated mechanisms for regulating the populations of their hosts.[28,56,69,135] In this chapter, we summarize the most important aspects of the main groups of known giant viruses of unicellular eukaryotes, including information about (1) the structure and composition of their virus particles, (2) the organization and content of their complex genomes, and (3) what we currently know about their replication cycles.

Classification and Evolutionary Relationships

In 2020, the International Committee on Taxonomy of Viruses (ICTV) presented a global organization and proposed a megataxonomy of the virus world.[77] Based on gene-sharing networks and phylogenetic analyses of hallmark genes, viruses of the NCLDV clade are now classified in the realm *Varidnaviria*, kingdom *Bamfordvirae*, phylum *Nucleocytoviricota*. In addition to the families *Phycodnaviridae*, *Mimiviridae*, and *Marseilleviridae*, several other groups of dsDNA viruses infecting unicellular eukaryotes belong to the nucleocytoviruses, including pandoraviruses, pithoviruses, molliviruses, orpheoviruses, cedratviruses, medusaviruses, faustoviruses, kaumoebaviruses, pacmanviruses (Fig. 17.2).[12,13,15,21,87,89,119,126,185] Most members of the kingdom *Bamfordvirae* encode a major capsid protein containing a double jelly-roll fold (MCP-DJR), forming pseudohexameric capsomers (Fig. 17.2). Each jelly-roll domain consists of two juxtaposed four-stranded beta sheets, and the icosahedral capsid lattice is made of vertical jelly rolls.[23,77] In addition, bamfordviruses encode other structure-related proteins, such as a single jelly-roll minor capsid protein and an FtsK-HerA–like ATPase involved in virus genome packaging, and replication enzymes, such as DNA polymerases and superfamily 3 helicases.[77] Remarkably, despite these structural and/or replication hallmark genes being shared and encoded by most bamfordviruses (including those not yet officially classified), some bamfordviruses have presumably lost a few of those hallmark genes or, in some cases, the original functions of the encoded hallmark proteins have been modified. These facts may explain, in part, why some protist-infecting viruses present atypical capsid shapes—such as the oval pandoraviruses, pithoviruses, cedratviruses and orpheoviruses, and the spherical molliviruses—in contrast to the icosahedral symmetry observed in most of the known viruses of this group (Fig. 17.1).[12,87,89,119]

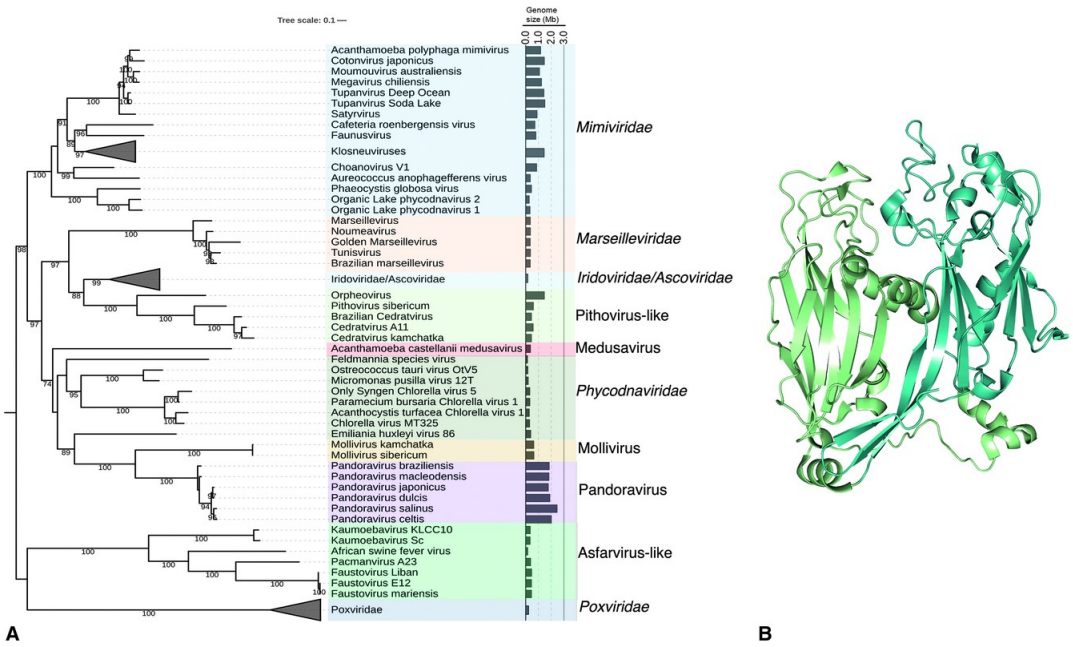


FIGURE 17.2 **Hallmark proteins of bamfordviruses**. **A**. Phylogenetic relationship within the phylum *Nucleocytoviricota* based on the polymerase B gene sequence. Representatives of all families included in the phylum were incorporated in this maximum likelihood tree. The genome size of each virus is shown in the *bar graph*. The genome size related to the collapsed clades is the mean value considering the viruses used to reconstruct the phylogenetic tree. The tree was built by using IQ-TREE with an evolutionary model LG+F+R5, selected by ModelFinder, based on the amino acid alignment of 72 viral sequences. Nodes have been labeled with bootstrap values. The scale bar represents the substitution rate. (Tree courtesy of Prof. Rodrigo Rodrigues, Universidade Federal de Minas Gerais, Brazil.) **B**. Structure of PBCV-1 MCP with the typical double jelly roll (PDB ID: 5TIP) shared by most of bamfordviruses. The N-terminal jelly-roll domain is colored in *pale green* and the C-terminal domain is colored in *light teal*. (Courtesy of Mateus Serafim, Universidade Federal de Minas Gerais, Brazil.)

The virions of nucleocytoviruses of unicellular eukaryotes are mostly nonenveloped, containing an inner lipid membrane.[1,77] The inner lipid membrane is usually related to the uncoating of the virion core into the host cell, by fusing with cellular lipid membranes, including the phagosome, endosome, or plasma membrane.[3,87,89,119] There are some notable exceptions. It seems that the faustovirus inner membrane has

been replaced by an inner protein capsid.[76] In tupanviruses, a long cylindrical tail is attached to an icosahedral capsid.[3] Moreover, in coccolithoviruses, an outer lipid envelope covers the capid.[92]

Considering the possible monophyletic origin of the viruses encoding the MCP-DJR structural module, it has been proposed that giant viruses evolved from smaller viruses, in a complex and long-term scenario[77,78]: (1) a tectiviruslike ancestor–infecting bacteria was incorporated by a proto-eukaryotic host during the endosymbiotic event that gave rise to mitochondria; (2) this tectiviruslike ancestor recombined with other mobile genetic elements, integrated into the host nucleus and evolved into the Maverick/Polinton-like elements. Most, if not all of these entities are likely more viral than transposons in their lifestyle; (3) after the acquisition of genes encoding RNA polymerase and capping apparatus, polintons escaped from the nucleus and then were able to replicate in the cytoplasm; (4) finally, after the acquisition/replacement of some genes and genome expansion, polintons gave rise to giant viruses. Interestingly, in this scenario, polintons are key players during the emergence and evolution of other viruses, including virophages, adenoviruses, and bidnaviruses.[77,78] Another theory, much more speculative and less detailed about the origin of giant viruses argues that they are descendants of a lost cellular domain and have faced reductive evolution.[33] Regardless of the giant virus origin, horizontal gene transfer, gene duplication, or even "*de novo*" gene creation have been described as evolutionary events that improve and speed up giant virus adaptation.[4,52,88]

Phycodnaviridae

As mentioned, electron microscopy observations of large VLPs in association with unicellular eukaryotic algae in the 1970s constitute some of the first potential records in the literature of the existence of giant viruses.[46,60,121,122] However, seminal studies on chloroviruses in the early 1980s represent a hallmark in the history of virology. For the first time, a large virus of a unicellular eukaryotic algae could be propagated, purified, and analyzed by classical virology methods, including the plaque assay standardized by James Van Etten and colleagues.[97,168,173,174] Since then, numerous studies on the structure, genomics, virus-host relationships, and even related to the biotechnological use of chlorovirus proteins have been carried out.[51,53,135,181]

Over the years, new eukaryotic algae viruses have been discovered, constituting what we know today as the family *Phycodnaviridae*, order *Algavirales*, class *Megaviricetes*. Metagenomic studies have revealed the diversity and ubiquity of these viruses in diverse environments.[70,146] According to the ICTV, six genera make up the family today: *Chlorovirus*, *Coccolithovirus*, *Prasinovirus*, *Prymnesiovirus*, *Raphidovirus*, and *Phaeovirus*. The last one encompasses viruses infecting multicellular brown algae. However, most phycodnaviruses infect unicellular eukaryotic algae, inhabitants of freshwater and sea, and are currently recognized as important players in aquatic communities, acting in algal bloom control, nutrient cycling, and carbon flow.[68,141] Among the hosts of phycodnaviruses are beautiful organisms, which establish complex symbiotic and ecological relationships in aquatic environments, such as *Emiliania huxleyi*, *Chlorella* spp., and *Ostreococcus* spp. However, with the discovery and phylogenetic analysis of new giant viruses of amoeba, it is increasingly evident that some genera currently classified in the family *Phycodnaviridae* must become new independent viral families.[19]

Virion Structure

Phycodnavirus virions consist of a 100- to 220-nm icosahedral outer shell and an inner bi-lipid membrane enclosing the genome and proteins in a dense core (Figs. 17.1 and 17.3).[170] An additional external membrane (envelope) was described in coccolithoviruses, a rare feature among unicellular eukaryote–infecting nucleocytovirus.[92] Among the phycodnaviruses, Paramecium bursaria Chlorella virus 1 (PBCV-1) has been the most structurally characterized. To date, a near-atomic analysis of the 190-nm capsid of PBCV-1 has been described, and it is the largest virus to be resolved at 3.5 Å resolution (Fig. 17.3). The PBCV-1 icosahedral capsid has a triangulation number of 169 (h = 8; k = 7), composed of trimeric capsomers arranged into 20 triangular and 12 pentagonal facets.[51] Three-dimensional (3D) reconstruction of cryogenic electron microscopy (cryo-EM) images revealed that one PBCV-1 vertex has a 560 Å long spike structure, of which 340 Å extend from the virus surface. This structure makes first contact with the host cell wall during infection (*see stages of replication*) (Fig. 17.3). The spike width is 35 Å at the tip and 70 Å at the base; this base is rooted inside the shell and extends 160 Å, allocated in a large pocket (a gap between the capsid and the inner membrane).[38] The PBCV-1 genome is not homogeneously distributed in the particle core due to this pocket. External fibers have also been described to extend from trisymmetron capsomers and might play a role in virus attachment to host cells. The capsid consists of 5040 major capsid protein copies, 60 copies of a penton protein and 1800 units of 13 different types of proteins. The minor capsid protein P2 acts as a tape-measure element, connecting fivefold vertices (Fig. 17.3).[51,167]

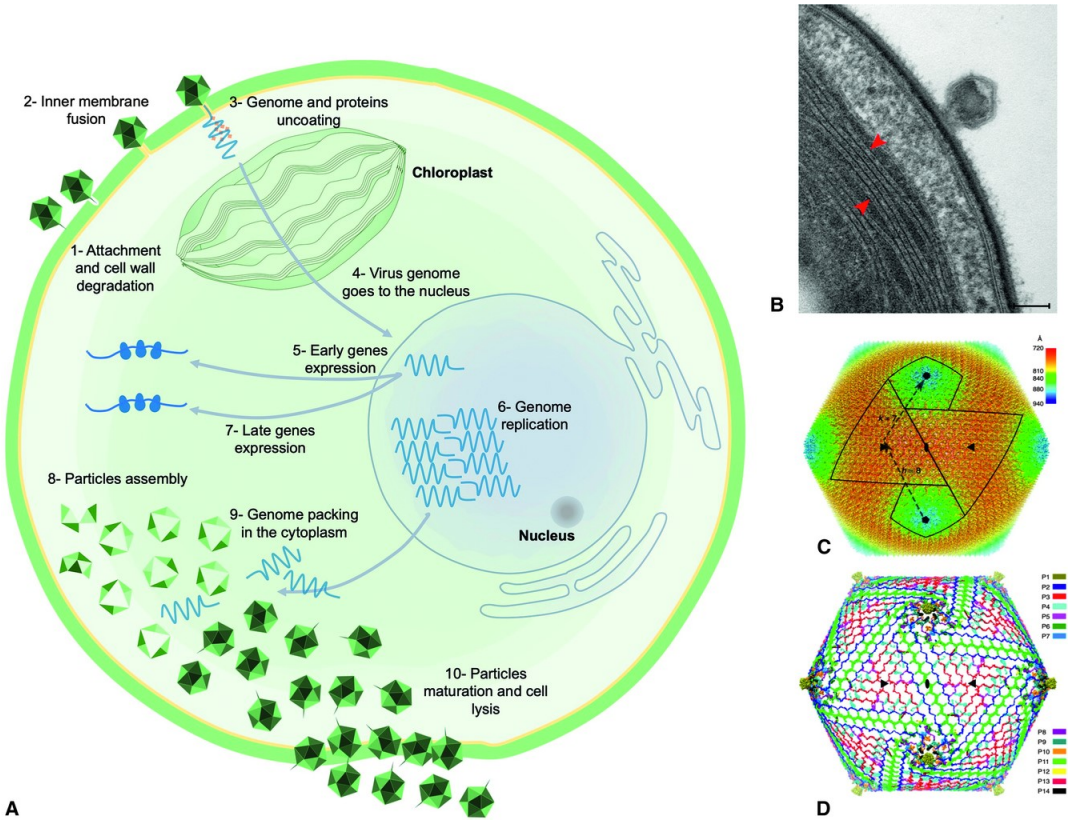


FIGURE 17.3 **Paramecium bursaria Chlorella virus 1 (PBCV-1) replication cycle and structural features**. **A**. Schematic overview of the PBCV-1 replication cycle. *1*—Infectious PBCV-1 particle attachment to the cell wall is mediated by the spike structure; wall degradation is probably promoted by the C-terminal portion of an alginate lyase–like protein present in the virion. *2*—The virion inner membrane fuses with the cell plasma membrane, forming a tunnel for uncoating. *3*—The virus genome and proteins are released into the host cytoplasm. *4*—The PBCV-1 genome moves to the algae nucleus. There is evidence that the virus promotes disruption of adjacent thylakoid membranes.[99] *5 and 6*—Early genes are expressed, including those related to genome replication, which occurs in the nucleus. *7*—Late genes are expressed, including those encoding structural proteins. *8-10*—In assembly sites, structural proteins are organized, forming empty particles. Those particles are filled with the virus genome in the cytoplasm. Particles are then released by lysis of the cell wall and plasma membrane. **B**. PBCV-1 virion attached to the cell wall. *Red arrowheads* show thylakoid membrane stacks adjacent to cell plasma membranes. There is evidence that adjacent thylakoid membrane disruption can be promoted by PBCV-1, paving the way for the virus genome to reach the nucleus. (Adapted from Milrot E, Shimoni E, Dadosh T, et al. Structural studies demonstrating a bacteriophage-like replication cycle of the eukaryote-infecting Paramecium bursaria chlorella virus-1. *PLoS Pathog*. 2017;13(8):e1006562.) **C**. Icosahedrally averaged cryogenic electron microscopy reconstruction of the PBCV-1 capsid. Colors indicate the distance from the center of the virus. The h and k vectors are indicated (used to calculate the T number). **D**. Cryogenic electronic microscopy density of PBCV-1 minor capsid proteins and the penton proteins after removing the outer capsid shell. The colors to the right indicate the several proteins present in the capsid. (**C** and **D** are adapted from Fang Q, Zhu D, Agarkova I, et al. Near-atomic structure of a giant virus. *Nat Commun*. 2019;10(1):388. http://creativecommons.org/licenses/by/4.0/.)

The proteome analysis of PBCV-1 virions indicated 148 virus-encoded proteins (from <10 to >200 kDa) and at least one host protein. A total of 106 proteins have unknown functions or homologs in databases (except for orthologs found in other phycodnaviruses).[117] PBCV-1 MCP (Vp54) seems to be totally glycosylated by virus-encoded proteins.[171] Besides the aforementioned major and minor capsid proteins, PBCV-1 virions carry signal transduction proteins, several membrane proteins, histone methylase, Cu/Zn superoxidase dismutase, and others.[117] Interestingly, a protein (A561L) containing a domain that resembles an alginate lyase was found in the virion: its C-terminal domain has *Chlorella* cell wall–degrading activity, strongly suggesting that this protein is essential during the chlorovirus entry phase.[6]

There are limited data on virion structure and composition regarding other phycodnaviruses. To date, the structure of Phaeocystis pouchetti virus, a prymnesiovirus, has also been resolved, revealing that the 220-nm icosahedral particles are composed of 2192 capsomeres arranged quasi-symmetrically with the triangulation number of 219 (h = 7; k = 10). A total of 60 fiberlike protrusions were described in association with the outer surface of the capsid; they may be important to virus attachment.[182] The proteomics of the E huxleyi virus (EhV), a coccolithovirus, revealed that its virion comprises 28 types of virus-encoded proteins, including MCP, lectin-binding proteins, thioredoxin, protein kinases, and >20 membrane proteins.[9] Additional studies on virion proteome and structure are necessary to unveil shared and exclusive features of phycodnavirus groups.

Genome Structure and Organization

Phycodnaviruses have dsDNA genomes ranging from 154 to 560 kb in size and a G/C content from 30% to 55%.[170] As mentioned previously, some representatives will probably be allocated to other viral families, especially those with the largest genomes.[19] A large number of phycodnavirus genome sequences are available, especially chloroviruses, coccolithoviruses, and prasinoviruses.

The chloroviruses have linear dsDNA genomes, 290-370 kb, encoding 330-416 predicted proteins so far.[167] As observed in other

nucleocytovirus, some chlorovirus proteins have multiple domains, expanding the functional repertoire encoded by the genome. The genome of PBCV-1 (331 kb with 416 coding DNA sequences [CDS]) has a terminal 35-nucleotide-long region covalently closed, forming hairpin loops.[191] Each hairpin is followed by a 2.2 kb inverted repeat sequence.[157] Intergenic regions are minimal and the coding regions of some genes overlap. Some core protein genes are located in clusters, named "gene gangs," which suggests selection for functionally related genes to remain in proximity.[150] In the central region of the genome, there is a polycistronic gene containing 11 transfer RNAs (tRNAs). Individual chloroviruses encode different tRNAs.[167] The intergenic regions of most of the early PBCV-1 genes contain the sequence AATGACA, preceding the start codon by ~100 nucleotides; however, there is no consensus promoter motif that has been identified for all early or late genes of PBCV-1.[59] Genome analysis of 36 chlorovirus genomes identified four distinct clades and an open pangenome, with 1345 clusters of orthologous genes, including 126 conserved in all viruses.[131] Comparison of the genomes of different chlorovirus host types revealed poor syntenic conservation.[150]

Interestingly, chloroviruses encode several remarkably small proteins well characterized, a factor that facilitated the characterization and even commercial use of some of these proteins: ATP-dependent DNA ligase, 5 mC and 6 mA DNA methyltransferase, site-specific DNA endonuclease, a type II DNA topoisomerase, and a tiny (82 amino acid) protein that forms a functional K^+ channel.[5,170] As mentioned previously, PBCV-1 encodes an extensive glycosylation machinery; some chloroviruses encode chitin synthase and enzymes involved in sugar metabolism, including UDP-glucose dehydrogenase, hyaluronan synthase, and glutamine/fructose-6-phosphate aminotransferase.[167,170,171]

The genome sequence of EhV strain 86 was announced in 2005, just a few years after its discovery in the English Channel.[177] Since then, other EhV genomes have been sequenced, revealing common features among coccolithoviruses genome.[10,107,108] Coccolithoviruses have linear 376-421 kb genomes, encoding 444-548 predicted proteins, with a G/C content of about 40%. At least four clades (A1, A2, A3, and B) have been identified among EhV strains, based on average nucleotide identity and phylogenetic analysis. Interestingly, EhV genomes are largely syntenic, even across different clades; the only exception is a region located between nucleotides 200 000 and 300 000, which presents several inversions, rearrangements, and substitutions.[108] Only ~90 EhV genes have functional predictions, including those related to a near-complete sphingolipid biosynthetic pathway that seem to be homologous to host (*E huxleyi*) genes.[177] Important EhV genes probably were acquired from eukaryotes, including those genes related to replication, repair, recombination, transcription, and nucleotide metabolism. Therefore, horizontal gene transfer seems to be an important feature in EhV evolution.[100] Three to six tRNAs were predicted in EhV genomes, including those common to all genomes: Arg, Asn, and Gln.[108]

The comparative analysis of viruses infecting the prasinophyte marine eukaryotic phytoplankton revealed that Micromonas pusilla virus genomes can harbor up to seven tRNAs, including five shared by all analyzed viral strains: The genomes of those strains are 173-205 kb, encoding 244-275 predicted proteins and a G/C content ranging from 39% to 43%. Only 80 clusters of orthologous genes are shared by all analyzed strains (core genome), including genes related to DNA replication, transcription, and structural proteins.[53] Ostreococcus tauri virus genomes also encode for tRNAs (eg, OtV-2 and OtV-5 encodes for five tRNAs), and some strains encode for cytochrome b5; DNA methyltransferase; and enzymes involved in sugar metabolism, lipid modification, and DNA methylation.[176] The comparative analysis of seven Ostreococcus lucimarinus viruses revealed ~190 kb genomes, with a G/C content of ~40%, encoding 243-269 putative proteins and five tRNAs. Some viral strains presented a 32 kb inversion in the central region of the genome.[44]

Although phaeoviruses infect multicellular algae, here, we will present some data for comparative purposes. Among the phaeoviruses, the genome of Ectocarpus siliculosus virus was announced in 2001, revealing a ~335-kb genome encoding 231 predicted genes.[43] Genes putatively involved in DNA integration were identified, which is compatible with the virus lifestyle and its latent phase. Repetitive elements, namely tandem repeats, represent 12% of the E siliculosus virus genome, and single-stranded DNA regions were described in the genome.[43] The sequence of Feldmannia virus strain 158 showed a ~154-kb genome encoding 150 predicted proteins, including those involved in DNA transposition, integration, replication, and methylation.[145] Finally, the 274-kb genome of Heterosigma akashiwo virus strain 53, a raphidovirus, encodes three tRNAs and ~240 proteins. Only 23 proteins had phycodnaviruses as best hits.[110] Therefore, the genomic data obtained in recent years have suggested that phycodnavirus consist of a genetically variable, heterogeneous, and polyphyletic group, encoding for a narrow set of shared universal genes (core) and deeply influenced by events of horizontal gene transfer with their hosts and other organisms. As aforementioned, the criteria to define the members of this family need to be revised.

Stages of Replication

Phycodnaviruses have been detected to infect a plethora of unicellular eukaryotic algae taxa as well as multicellular organisms. Most phycodnaviruses are lytic, but some can establish a latent phase after integrating the viral DNA in host genomes (eg, E siliculosus virus), being released only in the algal reproductive stage of life.[93,94,170] Furthermore, environmentally induced genetic and/or metabolic changes in host cells, including variations related to chromosomal euploidy, reproductive vs vegetative stages, and motile vs sessile forms, have been described as important factors regarding host susceptibility and permissiveness. In this regard, there are exciting data on the ecological roles of phycodnaviruses in aquatic environments.[95,101,116,165] Here, we present examples of the most studied viruses in the context of their replication cycle steps.

Virus entry in the host cell is quite variable depending on the phycodnavirus species. As mentioned previously, coccolithovirus and phaeovirus virions may have an external envelope. Therefore, EhV enters after envelope fusion with the host plasma membrane or endosomes.[92] Lipid rafts have been described as important for virus attachment and entry.[134,170] Despite the fact that EhV genomes encodes up to six RNA polymerase subunits, these proteins are not present in the virions; consequently, the transcription of virus early genes is promoted by the host machinery in the nucleus.[9] The enveloped E siliculosus virus infects wall-less host gametes via fusion of the virion envelope with the cell plasma membrane. The virus genome and associated nucleoproteins are directed to the cell nucleus within 5 minutes postinfection. Integration of virus DNA into the host genome is likely promoted by the integrase protein packaged in the virion. In the latent phase, the E siliculosus virus genome is propagated by mitosis in vegetative cells.[93,94]

The chlorovirus PBCV-1 attaches to the *Chlorella* cell wall, a process mediated by the virion spike (Fig. 17.3).[38,99] The punctiform degradation of the cell wall is probably promoted by the C-terminal portion of an alginate lyaselike protein present in the virion.[6] The inner membrane of the virion then fuses with the host membrane, forming a tunnel, where the virus genome linearly passes toward the host cytoplasm. Host plasma membrane depolarization is initiated by a virus K^+ channel present in the virion inner membrane, reducing cell turgor pressure, favoring the

release of the virus genome in the host cytoplasm, and blocking infection by other PBCV-1 virions.[5] The PBCV-1 genome, likely associated with proteins containing nuclear localization signals, moves to reach the nucleus, and there is evidence that the virus promotes disruption of adjacent thylakoid membranes.[99] In <10 minutes postinfection, there is transcription of virus early genes mediated by the host transcription–related proteins. The synthesis of the virus genome has been described to start either in the host nucleus (PBCV-1) or in the cytoplasm (EhV).[170]

Phycodnavirus virion assembly usually occurs in the host cytoplasm (Fig. 17.3). The PBCV-1 virus factories are composed of host cisternae derived from the endoplasmic reticulum. The cisternal membrane is cleaved into single bilayer membranes, which are associated with capsomers, forming empty capsids. DNA packing occurs at the periphery of viral factories.[98] Massive progeny formation has been described for most of the analyzed phycodnaviruses, filling the host cells with particles.[96] Interestingly, preceding complete cell lysis by 30-50 minutes, the partial disruption of the host cell membrane allows the release of part of the PBCV-1 progeny.[169] In contrast, the newly formed EhV particles move to the plasma membrane, and the envelope is acquired during budding of virus particles at ~4.5 hours postinfection (hpi).[92]

Mimiviridae

The aforementioned serendipitous discovery of APMV represents a scientific hallmark. For the first time in the history of virology, a 1 mega base pair (Mb) dsDNA genome had been described, encoding for proteins that were previously thought to be exclusive of cellular organisms.[124] The APMV virion has gigantic structure with icosahedral symmetry covered by long-associated fibrils, reaching up to 750 nm (Fig. 17.4); therefore, APMV particles are not filterable through 0.2-µm filters and can be visualized by optical microscopy.[82] This set of novel characteristics supported the establishment of a novel viral family named *Mimiviridae*, encompassing the genus *Mimivirus* and the species *A polyphaga mimivirus*. The family *Mimiviridae* belongs to the phylum *Nucleocytoviricota*, class *Megaviricetes*, and order *Imitervirales*. A few years later, the genus *Cafeteriavirus* was incorporated into the family, to encompass the species *Cafeteria roenbergensis virus* (CroV), a virus infecting a marine flagellate. A number of potential new species and strains have been described or reclassified as mimiviruslike entities, including klosneuvirus, tupanvirus, Bodo saltans virus, Aureococcus anophagefferens virus, Chrysochromulina ericina virus, Phaeocystis globosa virus, Tetraselmis viridis virus, and Prymnesium kappa virus, among others, revealing a considerable diversity in relation to virion structure, host spectrum, gene repertoire, and replication strategies.[3,14,22,27,36,42,74,137,140,147,148,156] The relationship of mimiviruses to smaller viruses called virophages has fueled debates on the definition of subviral agents.[58,83] In this section, we present a condensed view of the most important features regarding members of the family *Mimiviridae*.

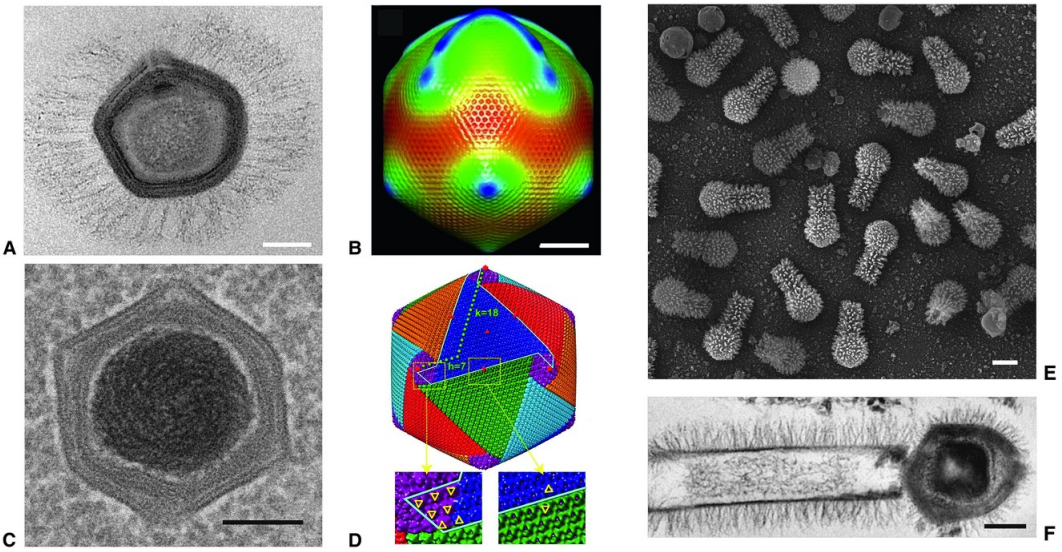


FIGURE 17.4 **The complex virions of mimiviruses**. **A**. Electron microscopy of mimivirus lineage A. Scale bar: 100 nm. (Courtesy of Isabella Aquino and Jônatas S. Abrahão, Center of Microscopy, Universidade Federal de Minas Gerais, Brazil.) **B**. Cryo-EM reconstruction of APMV. Scale bar: 100 nm. (Adapted from Xiao C, Kuznetsov YG, Sun S, et al. Structural studies of the giant mimivirus. *PLoS Biol*. 2009;7(4):e1000092. http://creativecommons.org/licenses/by/3.0/.) **C**. Thin-section electron microscopy image of CroV. Scale bar: 100 nm. (Courtesy of Ulrike Mersdorf and Matthias Fischer, MPI for Medical Research, Heidelberg, Germany.) **D**. Reconstruction of the CroV capsid. The isosurface of the map was colored by pentasymmetrons (*purple*) and trisymmetrons (*blue*, *red*, *green*, *cyan*, and *orange*). One of the 30 edges of the icosahedron is marked with a *cyan line*. Two surface areas are magnified, and selected capsomers are labeled with *yellow triangles* to show their orientations. (Adapted from Xiao C, Fischer MG, Bolotaulo DM, et al. Cryo-EM reconstruction of the Cafeteria roenbergensis virus capsid suggests novel assembly pathway for giant viruses. *Sci Rep*. 2017;7(1):5484. http://creativecommons.org/licenses/by/4.0/.) **E, F**. Scanning and transmission electron microscopy of tupanvirus soda lake particles. Scale bars: 500 nm (panel **E**) and 200 nm (panel **F**). (Adapted from Abrahão J, Silva L, Silva LS, et al. Tailed giant Tupanvirus possesses the most complete translational apparatus of the known virosphere. *Nat Commun*. 2018;9(1):749. http://creativecommons.org/licenses/by/4.0/.)

Virion Structure

Members of the *Mimiviridae* family are variable in their size, composition, the presence of surface fibrils, and the tail (Figs. 17.1 and 17.4). Among cultivated mimiviruses, virions range from ~150 nm in diameter (*Phaeocystis globosa virus* I) to 1200 nm (tupanviruses) in length.[3,24] Since the discovery of APMV, several techniques, as thin-section electron microscopy, cryo-EM, atomic force microscopy, STEM tomography, and others, have been applied to better understand its structure, composition, and life cycle.[80,104,105,143,149,180,187] The APMV virion is composed of a 500-nm outer capsid shell covering an inner capsid shell, an inner membrane, and a core wall containing the genome and other

elements (proteins and tRNAs) related to early steps of viral replication cycle. The outer capsid shell has icosahedral symmetry and comprises MCP trimers (the L425 gene). The capsid has 20 faces and an exit portal structure in 1 of the 12 vertices, named stargate (Figs. 17.4 and 17.5).[143,180,187] This structure opens during the early steps of the virus cycle, ultimately promoting the viral core/seed transfer to the host cytoplasm (Fig. 17.5).[11] The stargate portal is sealed by a proteinaceous complex named starfish (Fig. 17.5).[143,180,187] The product of APMV gene L454 probably acts as a tape-measure, connecting fivefold vertices beneath the outer MCP layer,[178] and the APMV protein L410 is an abundant component of the viral core.[125] The APMV triangulation number was not determined due to limited resolution of structural studies, but it is estimated to be 972-1200 (h = ~19; k = ~19).[180] However, the triangulation numbers of other *Mimiviridae* members have been obtained: the A anophagefferens virus triangulation number is 169 (h = 7; k = 8),[65] and CroV has the highest triangulation number determined so far: 499 (h = 7; k = 18), with 4980 capsomers and 14 940 MCP copies (Fig. 17.4).[179]

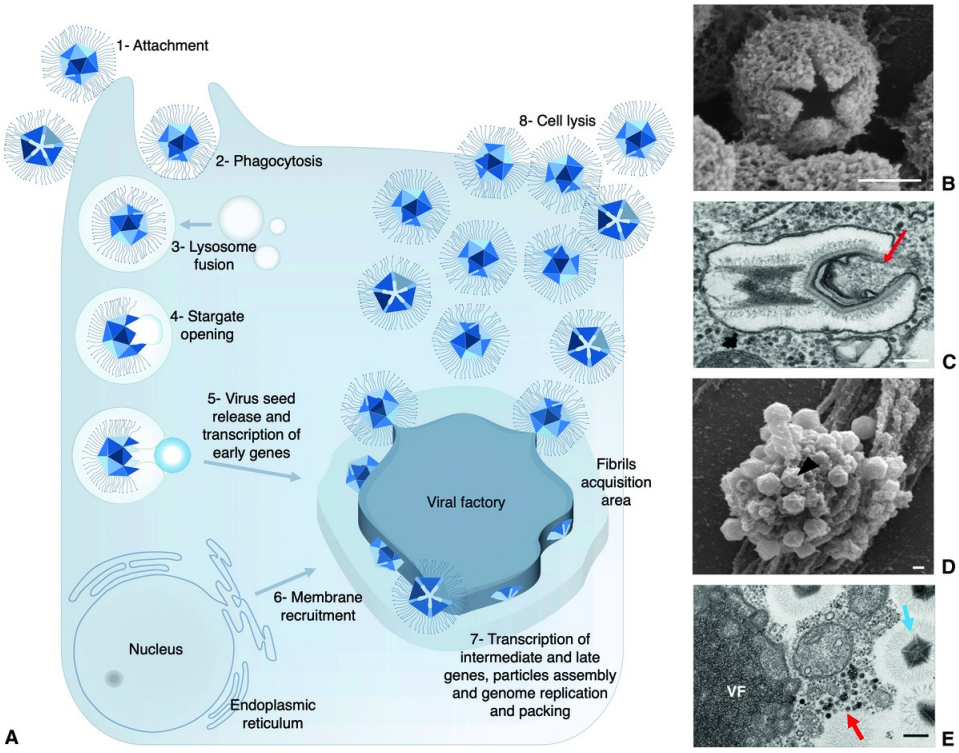


FIGURE 17.5 **Mimivirus replication cycle and structural features**. **A**. Schematic overview of the APMV replication cycle. *1 and 2*—APMV infectious particle attachment to the cell surface is mediated by viral fibrils, triggering phagocytosis. *3-5*—Lysosomal fusion with phagosome-containing APMV promotes acidification, leading to stargate opening and release of the viral seed into the amoebal cytoplasm. APMV early genes are then expressed by a transcriptional set of proteins present in the virion. *6 and 7*—The viral seed gives rise to a mature and micrometric viral factory. Membrane recruitment from the endoplasmic reticulum to the viral factory is involved in the initial steps of particle morphogenesis. Intermediate and late genes are expressed, the genome is replicated, and particle assembly occurs sequentially in the periphery of the viral factory. The virus genome is packaged into the particles at a site opposite to the stargate vertex. Fibrils are incorporated into the particle surface concomitantly with genome packaging, in a region named the fibril acquisition area. Newly formed mature particles fill the amoebal cytoplasm and are released by cell lysis. **B**. Scanning electron microscopy of a tupanvirus with an open stargate. Scale bar: 200 nm. (Courtesy of Prof. Juliana Cortines, Universidade Federal do Rio de Janeiro/University of Connecticut.) **C**. Transmission electron microscopy of tupanvirus uncoating in the amoebal cytoplasm. The *red arrow* shows the fusion of the viral inner membrane with the phagolysosomal membrane, after stargate opening. Scale bar: 450 nm. (Adapted from Abrahão J, Silva L, Silva LS, et al. Tailed giant Tupanvirus possesses the most complete translational apparatus of the known virosphere. *Nat Commun*. 2018;9(1):749. http://creativecommons.org/licenses/by/4.0/.) **D**. Scanning electron micrograph of a tupanvirus factory after *Vermamoeba vermiformis* infection. The *arrowhead* shows the starfish structure of a particle on the surface of the factory. Scale bar: 300 nm. (Adapted from Silva LCF, Rodrigues RAL, Oliveira GP, et al. Microscopic analysis of the tupanvirus cycle in Vermamoeba vermiformis. *Front Microbiol*. 2019;10:671. http://creativecommons.org/licenses/by/4.0/.) **E**. Transmission electron microscopy of the viral factory of a lineage A mimivirus. The *blue arrow* shows a mimivirus particle. The *red arrow* shows newly formed particles of the Sputnik virophage, replicating in association with the mimivirus factory. Scale bar: 200 nm. (Courtesy of Bruna Azevedo and Jônatas S. Abrahão, Center of Microscopy, Universidade Federal de Minas Gerais, Brazil.)

The surface of the APMV outer capsid shell, except for the starfish structure, is covered with 140-nm-long fibrils, which give the virion a hairy appearance and expand its size to up to 750 nm (Fig. 17.4).[104,132] These surface fibrils are variable in size, thickness, abundance, and organization, depending on the mimivirus.[3,18,132] The fibrils consist of multiple components, including *N*-acetylated viosamine and gene products R135, L725, L829, and R856.[32,120,154] Fibrils are capped with an ellipsoidal protein head of 25 kDa.[81] Fibrils are glycosylated and have been referred to as a peptidoglycanlike containing structure, which favors the affinity of APMV particles for crystal violet during Gram staining. It has been hypothesized that fibrils are important but nonessential elements to promote virus attachment in host cells and may act as "bait" to optimize virion internalization by triggering phagocytosis in amoebas.[132] Cotonvirus japonicas fibrils are denser and shorter than those of APMV,[163] and in Megavirus chiliensis, some fiber bundles called cowlicks are longer than the rest.[18] No fibrils were observed on the surface of CroV virions[179] (Fig. 17.4).

Tupanvirus soda lake and tupanvirus deep ocean are tailed mimiviruses.[3] However, in contrast to some bacteriophages, the tupanvirus tail is not a strawlike structure that promotes the injection of the virus genome into host cell compartments during virus entry. In fact, tupanvirus has

a capsid that is structurally similar to APMV, including the stargate vertex. The 550-nm-long and 450-nm-thick cylindrical tail is attached to the capsid at the vertex opposite of the stargate vertex (Fig. 17.4). Tupanvirus capsid and tail are covered by a shorter, thicker fibril layer. Both the capsid and tail content are released in amoeba upon infection. The tupanvirus tail is variable in length, reaching up to 2300 nm.[3,152]

A total of 236 different proteins were found in APMV particles,[61] including structural proteins (eg, major capsid protein and major core proteins), several RNA polymerase subunits, mRNA capping enzymes, helicases, and other transcriptional elements; proteins involved in redox pathways that may be important in the context of the amoeboid phagosomal environment during virus entry; and DNA repair, which presumably acts on viral DNA even inside the particle. Some mRNAs have been found in APMV particles, including those related to aminoacyl tRNA-synthetases (aaRS), DNA polymerase, and TFII-like transcription factor.[41] CroV, tupanvirus, and A anophagefferens virus particle proteomics have indicated 141, 187, and 43 proteins, respectively, some of them with functions similar to those described for APMV particles.[57,65,143] Future studies targeting specifically the composition of the tupanvirus tail will be useful to understand the role of this structure in early steps of the cycle.

Genome Structure and Organization

The members of the *Mimiviridae* family have dsDNA genomes ranging from 371 kb in A anophagefferens virus to 2.12 Mb in Yasminevirus (Fig. 17.1). The genomes are mostly linear, although the genome of T viridis virus has been described as circular. The G/C content ranges from 23.3% in CroV to 41.2% in T viridis virus and is usually lower than the G/C content of their protist hosts.[55,148] Different methods have been used to predict the number of genes in each cultured mimivirus genome, but current information indicates that it ranges from 377 in A anophagefferens virus to 1541 in yasminevirus.[22,64] In APMV and other *Mimiviridae*, introns or inteins have been described in association with genes encoding the major capsid protein, DNA polymerase, DNA topoisomerase, or RNA polymerase large subunit.[30,41,109] As observed in other DNA viruses, variable and nonessential genes are more likely to be found at the termini of the genome. A Q-like topology model has been proposed to explain APMV genome replication, based on the pairing of 617 bp inverted repeats.[40] Putative promoter motifs have been described, including the AAAATTGA (early) and TCTA (late), but variations may be found in different mimiviruses.[160]

A substantial number of predicted genes with no recognizable homologs in databases, usually referred as ORFans, have been identified in mimivirus genomes; many of them are shared by several strains and encode for proteins also present in the virions.[41,61,124] As mentioned previously, *Mimiviridae* members may encode for proteins that are rare or have never been seen in the virosphere. A trademark among mimivirus genomes is the presence of translation-related genes, including aaRS, tRNAs, and translation factors (initiation, elongation, and termination).[3,22,41,55,124,147] Sequencing of the APMV genome revealed the presence of four types of aaRS-coding genes.[124] aaRS promotes the attachment of a given amino acid to cognate tRNA. Following the characterization of APMV, several mimivirus isolates have been described, including lineage A mimiviruses (including APMV), lineage B (moumouviruslike viruses), and lineage C (megaviruslike viruses), with slight variations in the number and variety of aaRS genes, including paralogous genes with distinct evolutionary histories.[18,29,124,183] However, in 2017, the metagenomic analysis of a wastewater treatment plant in Austria revealed up to 19 aaRS in the genome of klosneuviruses, a group of mimivirus relatives.[147] In the following year, a complete set of 20 aaRS and up to 70 tRNAs, corresponding to all 20 amino acid specificities, was found in tupanvirus genomes.[3] Subsequently, a similar profile was observed in minevirus.[22] Conclusions about mimivirus aaRS gene origins based on phylogenetic analyses are controversial. Tracking aaRS gene origins has been challenging because some mimivirus aaRS genes belong to deep-branching viral clades, while others seem to have been acquired more recently from cellular organisms. Those aaRS genes seem to have been obtained by mimiviruses in different scenarios and evolutionary occasions.[3,4,147] The reason why some mimivirus have conserved an extensive set of aaRS and other translation-related genes needs further investigation, but perhaps the retention of translation-related genes in viral genomes could be advantageous for exploring new hosts with different codon and amino acid usages: tupanviruses, for example, are able to infect both *Vermamoeba vermiformis* and different species belonging to the *Acanthamoeba* genus. In addition, keeping translation-related genes would be an advantage in when host translation system is shut-off as viral defense.[3,152]

Other unexpected genes have been found in different putative members of *Mimiviridae*. Tupanviruses encode a putative citrate synthase, an enzyme belonging to the tricarboxylic acid cycle, probably acquired from a sympatric bacterium by horizontal gene transfer.[129] Genes putatively encoding enzymes related to the biosynthesis of 3-deoxy-d-manno-octulosonate, involved in carbohydrate metabolism, were found in CroV.[55] Key genes related to mannitol metabolism and fermentation were found in T viridis virus, including mannitol 1-phosphate dehydrogenase, alpha-galactosidase, pyruvate formate-lyase, and pyruvate formate-lyase–activating enzyme.[148] Genes encoding rhodopsin, a protein that captures or senses sunlight, were found in the metagenome of Choanovirus, a member of the extended *Mimiviridae*.[106] Myosin, actin, and kinesin genes have been found in giant virus genomes by metagenomic and metatranscriptomic studies.[69,75] Therefore, as the knowledge on mimiviruses expands, a more sophisticated and complete repertoire of genes will be incorporated into the family pangenome.

Stages of Replication

Mimiviridae members are able to infect a broad range of heterotrophic or autotrophic eukaryotes, including amoeba such as *Acanthamoeba* sp. (eg, APMV, Tupanvirus, Cotonvirus) and *V vermiformis* (eg, Tupanvirus, Yasminevirus, Fadolivirus); the marine flagellate grazer *Cafeteria* sp. (CroV); the kinetoplastid flagellated phagotrophic protozoa *Bodo saltans* (Bodo saltans virus); the bloom-forming phytoplankton *Phaeocystis globosa* (Phaeocystis globosa virus group I); the heterokont alga *Aureococcus anophagefferens* (Aureococcus anophagefferens virus); other phytoplankton species such as *Chrysochromulina* (Chrysochromulina ericina virus) and *Tetraselmis viridis* (Tetraselmis viridis virus); and choanoflagellates (Choanovirus). Mimiviruses have been found widespread in oceans, spring water, soil, and even as members of animal microbiomes.[3,24,27,36,42,55,74,82,137,140,146,148,156,163] The association between mimiviruses and disease in vertebrates is controversial and requires further investigation.[2,84,139,190]

In contrast to other DNA viruses, such as vaccinia virus, studies on the replication of APMV and other giant viruses of amoebae are still in the descriptive phase, and the molecular mechanisms that explain virus entry, morphogenesis, and release remain unknown. The enormous number of mimivirus genes, the need for a robust gene knockout system, and the scarcity of reagents and antibodies applicable to protists cells represent some of the challenges that the giant virus scientific community will need to face to elucidate molecular mechanisms.

Fibrils may optimize the adhesion of APMV to *Acanthamoeba castellanii* cells mediated by glycans, specifically mannose and

N-acetylglucosamine. The absence of fibrils does not prevent infection, but virus adhesion to host cells seems to stimulate the formation of membrane protrusions and particle intake.[132] It is very likely that APMV-related strains, Yasminevirus, Tupanvirus, and other close relatives enter the host cells by the phagocytic pathway (Fig. 17.5). Consistently, the treatment of amoebas with cytochalasin D reduced APMV particle intake.[11] Amoebas can ingest one or several APMV particles, and there is increasing evidence that phagosome acidification is a stimulus for stargate opening.[11,143,187] Vesicles containing electron-dense content, probably lysosomes, fused with phagosomes containing APMV particles in *A castellanii* cells.[11] After acidification, the starfish seal is removed, resulting in the stargate opening[143,162,187] (Fig. 17.5). After the stargate opening, the virion inner membrane fuses with the phagolysosome membrane, forming a tunnel through which the viral core is transferred to the cytoplasm.[11,105] Tupanvirus capsid-core release is very similar to that described to APMV, but the tail content is released by an unknown process.[3] The entry and uncoating mechanisms of other family members may differ from APMV entry, for instance in cell-walled or flagellated hosts.

APMV gene transcription is temporally regulated, and early genes start to be expressed immediately after core release (Fig. 17.5).[105] The core, also known as the viral seed, expands in complexity and size in the cytoplasm, forming a >1.5-µm viral factory.[105,162] As mimivirus particles carry a complete set of transcriptional related proteins, it is believed that all steps of the replication cycle occur in the cytoplasm.[61,143] However, the role of nuclear factors in the APMV cycle needs further investigation. The virus factory is roughly oval shaped, nonmembrane bound, contains a large electron-dense center, and is surrounded by an electron-lucent fibrillar area. Ribosomes often surround the viral factory but are mostly excluded from it, raising questions regarding the regulatory signals used by APMV to recruit newly synthesized viral proteins to the factory.[11,105,162] APMV DNA replication and repair occur in the factory, promoted by an extensive set of viral proteins.[61,143] The virions are assembled in well-defined, sequential zones.[11,105,162] Endoplasmic reticulum membranes are transported to the viral factory, arranged as multivesicular bodies, and then modified to form membrane sheets. During particle assembly, these membranes associate with nascent capsid protein lamellae.[11,104,158] The incorporation of new capsomers probably occurs in a spiral fashion around a pentameric vertex.[179] Nascent capsids appear at the surface of the viral factory, with the stargate facing away from the center of the viral factory.[11,61,152] The genome is packaged at a capsid opening opposite of the stargate region. In tupanviruses, the tail is attached in the same region as genome packaging occurs, but it is unknown if and how the two processes are related.[187] Concomitantly, fibrils are attached to the capsid surface at the periphery of the viral factory, in a zone called the fibril acquisition area (Fig. 17.5).[11] After maturation at 8 hpi, particles accumulate in the amoebal cytoplasm, surrounding the viral factory and eventually filling the entire cytoplasm; they are subsequently released by cell lysis.[11] It is noteworthy that tupanvirus-infected cells often aggregate with uninfected cells, a process mediated by expressing a mannose-binding protein on the cell surface. Large aggregates of cells, named bunches, are formed and might be related to the optimization of virus dissemination.[113]

Virophages

Following the discovery of mimiviruses, the description of associated, smaller viruses called virophages added even more complexity to the protist-infecting virus universe.[58,83] Virophages are *la*rge *vi*rus-*d*ependent or -*a*ssociated *vi*ruses classified in the family *Lavidaviridae*. This family belongs to the phylum *Preplasmaviricota*, class *Maveriviricetes*, order *Priklausovirales*.[77] Virophages have 50- to 70-nm icosahedral membrane-less capsids, with a triangulation number of 27, mostly formed by MCP in association with a minor capsid protein, also known as penton protein.[58,83,161,189] In addition, virophages encode other morphogenesis proteins, such as a cysteine protease and a Fts-HerA–like ATPase. The virophage genomes are dsDNA, linear or circular, ranging from 17 to 30 kb, encoding not only the aforementioned structure-related proteins but also a DNA replication module.[49] In addition, recombinases and integrases may be found in virophage genomes, which is compatible with the fact that virophages have been found integrated in giant viruses or even in host-cell genomes (provirophages).[45] Virophage sequences have been found in several metagenomic surveys and seem to be important ecological players in natural environments, balancing the levels of giant virus populations.[136]

The virophages Sputnik and Zamilon were described in association with different mimiviruses, co-infecting *A castellanii*[45,63,83,102]; the virophage mavirus was described in association with CroV, co-infecting *C roenbergensis*.[58] Those host cells are not permissive to virophage replication by themselves. Therefore, virophages replication cycle is dependent on the presence of a compatible giant virus. Virophages are presumably dependent on the transcriptional machinery of giant viruses. This hypothesis is strongly supported by the fact that virophages have transcriptional signals very similar to those found in their associated giant viruses, including late promoter motifs.[40,58,86] Interestingly, the transcription of virophage genes starts during the late steps of the associated giant virus cycle. Newly formed virophage particles are assembled within giant virus factories (Fig. 17.5) and released after cell lysis and potentially also by exocytosis.[45,73,83]

The impact of virophage replication on the final titers of the associated giant virus varies. Mavirus, for example, reduces CroV replication by about 99%[58]; some sputniklike virophages reduce mimiviruses progeny by ~66%-90%.[83] Zamilon virophage replication, however, has no impact on lineage B and C mimivirus progeny formation.[63] It is worth noting that the deletion of 81 nucleotides at ORF 8 of Guarani (a sputniklike virophage) results in a broader virophage host range, allowing Guarani to replicate in association with not only APMV strains but also tupanviruses.[103] Remarkably, the mavirus genome can integrate in the DNA of the cellular host *C roenbergensis*, a phenomenon likely mediated by the virophage-encoded integrase. When such a cell is infected by the lytic CroV, provirophage genes are transcribed and translated, resulting in the release of virophage particles and protection of the *C roenbergensis* population.[56] On the other hand, a mimivirus lineage A defense system against Zamilon virophages called MIMIVIRE has been described. Although the mechanisms need further investigation, the gene R349 has 28 or 15 nucleotide repeats similar to regions found in the Zamilon genome, and it was hypothesized that it guides a system analogous to CRISPR-Cas to block virophage replication.[90] In addition to virophages, mimiviruses have been associated with mobile genetic elements called transpovirons.[45,73] These elements are 7 kb long, linear dsDNA, encoding 6-8 genes including a transposase and a helicase, and have been found in association with all lineages of mimivirus. The phylogenetic similarity among transpovirons subclades found in association with each mimivirus lineage suggests that these mobile genetic elements established dependence on mimiviruses before evolutionary radiation.[73]

Marseilleviridae

The discovery of mimiviruses prompted the search for other viruses of amoebae. In this context, in 2007, a new viral entity was isolated from a water sample collected in a cooling tower located in Paris, France.[34] This virus, isolated in *A polyphaga*, was named marseillevirus, referring to the city where this virus was first isolated (Aix Marseille Université, Marseille, France). Since its discovery, dozens of marseilleviruses have been

described in different continents and from a plethora of substrates, both by isolation methods and by metagenomic studies.[16,20,47,48,50,138,164] Currently, five different marseillevirus lineages have been described (A-E). The 230-nm icosahedral marseillevirus particle contains a circular dsDNA genome that is up to 404 kb in length (Fig. 17.1).[138] The marseilleviruses are classified in the class *Megaviricetes*, order *Pimascovirales*, family *Marseilleviridae*, genus *Marseillevirus*.[77] The viruses of this group highlight the sympatric lifestyle of parasites and symbionts of amoebas, which resulted in the mosaic profile of marseillevirus genomes.[34]

Virion Structure

Studies using thin-section and cryo-EM 3D reconstruction, coherent diffractive imaging, cryo-tomography, and single particle analysis of different marseillevirus isolates, especially melbournevirus (a lineage A marseillevirus), have revealed that the particles have icosahedral shape, with a diameter of ~230 nm and a 8.4-nm-thick capsid shell (Fig. 17.6).[34,91,111] The triangulation number is 309 (h = 7; k = 13), constructed by 3080 pseudo-hexagonal capsomers, which are assembled from 9240 MCP molecules. Ordered fibrous protrusions that are 7.5 nm in diameter and 12 nm in length with globular ends were described on the particles surface. A 3-nm-thick inner membrane surrounds the genome and proteins in a central region of the particle.[111] The composition of this membrane still needs further investigation, but Western blot assays of the particles indicated the presence of the endosomal protein SNX2.[17] A structure called "large and dense body" (30 nm) was consistently observed in all melbournevirus particles, close to the inner membrane; however, its function is not yet known.[111]

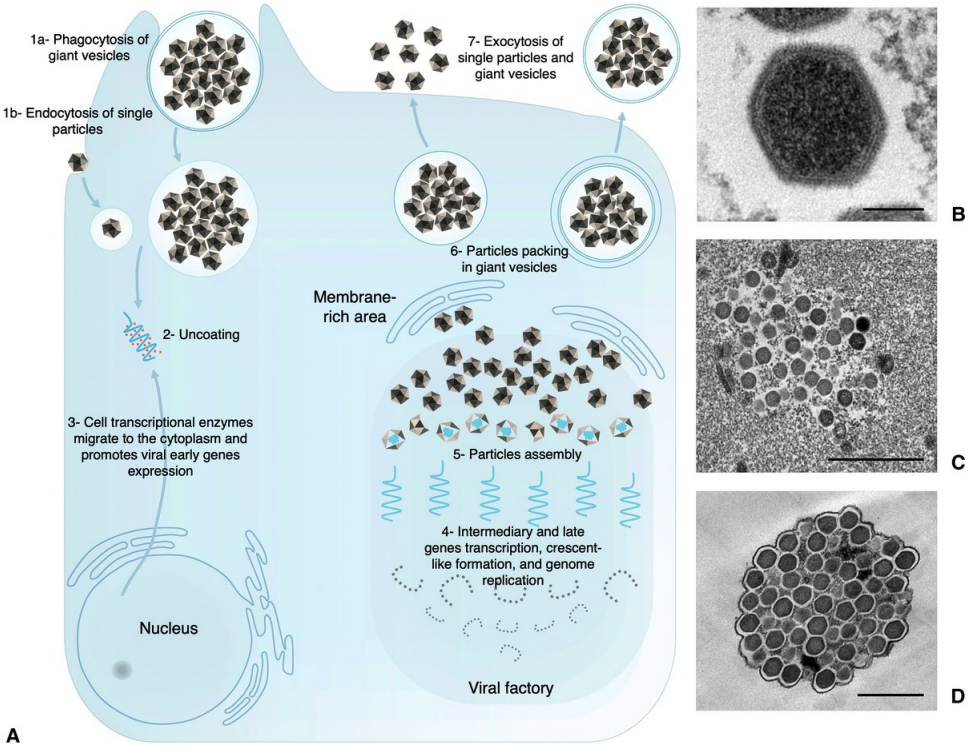


FIGURE 17.6 **Schematic overview of the marseillevirus replication cycle**. 1a—Marseillevirus particles attach to the cell membrane and likely trigger clathrin-mediated endocytic pathways. 1b—Alternatively, giant vesicles containing several marseillevirus particles trigger the phagocytic pathway. 2 and 3—Particles release the viral genome and proteins into the host cytoplasm. Virus early gene transcription is mediated by host enzymes recruited from the nucleus. 4—In the viral factory, intermediate and late genes are transcribed, followed by crescentlike formation and genome replication. *5*—Structural and genomic components are assembled concomitantly in mature particles and migrate to a membrane-rich area, at the periphery of the viral factory. 6—Part of the newly formed particles are wrapped into large and giant vesicles, containing from one to several membranes. 7—Single particles and giant vesicles containing many particles are exocytosed. Eventually, the host cell lyses, releasing more particles. **B**. Transmission electron microscopy of a marseillevirus lineage A particle. Scale bar: 100 nm. **C**. Transmission electron microscopy of a region of marseillevirus factory. Scale bar: 1000 nm. **D**. Transmission electron microscopy of marseillevirus giant vesicle after exocytosis. Scale bar: 500 nm. (Images in panels **B**-**D** are courtesy of Talita Machado and Jônatas S. Abrahão, Center of Microscopy, Universidade Federal de Minas Gerais, Brazil.)

Proteomic analyses of different marseillevirus isolates show marked differences in relation to the number of proteins detected in the particles, from 49 to 283 proteins (including proteins incorporated from the amoebal host). This discrepancy is probably due to methodological differences, including the purification of particles, sensitivity, and analysis of the results.[34,50] Nevertheless, MCP, usually the most abundant protein in the virions of other icosahedral viruses, is ranked third and fourth in the marseillevirus isolates noumeavirus (NMV_116) and melbournevirus (MEL_305), respectively. The most abundant protein in the marseillevirus particles is a 150 amino acid hypothetical protein, which is highly conserved among all isolates. Other proteins found in high abundance in marseillevirus particles are three virus-encoded histonelike proteins.[50] The analysis of the marseillevirus fused histone pairs Hβ-Hα and Hδ-Hγ show strong structural analogy with the eukaryotic histone pairs H2B-H2A and H4-H3, being virtually identical to canonical eukaryotic nucleosomes.[166] Therefore, it is very likely that those abundant virus-encoded histonelike proteins act by condensing viral DNA during encapsidation. Host proteins associated with oxidative stress, such as peroxidases and thioredoxins, have been found in marseillevirus particles and may be important in the context of the endosome or phagosome environment during virus entry.[34,50]

Genome Structure and Organization

The amoebal intracellular environment promotes a sympatric lifestyle for many types of organisms, including viruses, bacteria and other eukaryotes. In this environment, gene transfer can occur among amoebas, their parasites and symbionts. Lateral gene transfer has been described to be important for giant virus evolution, and this feature has also been observed in marseillevirus genomes. Genes putatively acquired from bacteria, eukaryotes and other viruses seem to contribute to the marseillevirus genetic repertoire, and this feature has led to the proposal that amoebas are “melting pots” of microbial evolution.[34,138]

Marseillevirus genomes are circular dsDNA ranging from 340 to 404 kb and a G/C content of about 43%.[26,138] However, metagenomic data indicate that the genomes of marseillevirus relatives can reach up to an impressive 763 kb and a G/C content of 62.3%, suggesting that marseilleviruses might have diversified and adapted to unknown niches and hosts.[20] Considering the marseillevirus strains discovered by isolation methods only, the number of predicted ORFs range from 386 to 550.[26,138] As observed for most giant viruses, most (59%) of the predicted ORFs of marseillevirus isolates had no homologs in databases (ORFans). However, marseillevirus genomes contain NCLDV core genes and are thus part of the *Nucleocytoviricota* (Fig. 17.2).[34] Translation-related genes are absent among the isolated marseilleviruses, in contrast to the extensive arsenal found in members of the family *Mimiviridae*. However, several classes of aaRS and tRNAs were attributed to be part of marseillevirus in metagenomic sequences.[20]

Paralogous genes are abundant in marseillevirus genomes (from 18% to 31% of the predicted ORFs), especially 20 genes encoding proteins containing membrane occupation and recognition nexus (MORN) repeats, which may promote important steps during viral morphogenesis and release. Serine-threonine kinase paralogs have also been described, and their encoded proteins may act in cell signaling. Several copies of predicted proteins containing the PFAM02399 domain have been detected, and they likely participate in the replication of the viral circular genome.[34]

Comparative analysis of 12 marseillevirus isolates suggested a bias in genome sequence conservation. Considering the MCP gene as the starting point of such circular genomes, the rightmost parts of the genomes are more conserved at the nucleotide level, regardless of the marseillevirus lineage. Core genes are more frequently found in this conserved region. Genetic inversions and rearrangements are more frequently observed at the leftmost two-thirds of the genomes.[26]

The AT-rich motif AAATATT is widespread in intergenic regions of all marseillevirus lineages, in association with 55% of the predicted genes. Different single nucleotide polymorphisms (SNPs) in the AAATATT motif impact experimental gene expression.[115] However, RNA-seq analysis of marseillevirus-infected amoebas was not able to associate this motif with any class of temporally expressed genes, suggesting that other sequences and elements may control the expression of early, intermediate, and late transcripts.[130]

Stages of Replication

Although marseilleviruses were discovered after inoculating environmental samples into cultures of *A polyphaga*, these viruses are also capable of infecting *A castellanii*.[17,34] In both cells, marseilleviruses induce cytopathic effects, including rounding, cell detachment, and lysis. Some lineage B marseilleviruses can induce cell aggregation, forming “bunches,” similarly to those described for tupanviruses, but in this case, cell interaction seems to be mediated by galactose.[16,113]

Marseilleviruses have multiple entry pathways in *A castellanii* (Fig. 17.6). Individual virus particles trigger an endosomal acidification-dependent pathway, possibly via clathrin-coated endosomes.[17] Pretreatment of amoebas with chloroquine negatively impacts virus replication. In addition, marseilleviruses can enter amoebas as collective infectious units, called giant vesicles. Such vesicles are formed at the end of the replication cycle and are then released to the extracellular environment by lysis or exocytosis. The giant vesicles can reach >1000 nm in diameter, containing from a few to thousands of marseillevirus particles. Most of those vesicles present two membrane layers surrounding the particles, although vesicles with one, three, or even more membrane layers have also been described. Giant vesicles seem to trigger the phagocytic entry pathway, because virus entry and replication are negatively impacted after pretreating *A castellanii* with cytochalasin D but not with 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) or chloroquine.[17] However, due to the poorly characterized activity of pharmacological inhibitors in amoeba, more studies are needed to confirm whether the aforementioned pathways are indeed activated by marseillevirus particles and giant vesicles. Clusters of aggregated marseillevirus particles (not wrapped by membranes) also seem to trigger the formation of pseudopods.[17]

The mechanisms related to uncoating giant vesicles containing marseillevirus particles after being phagocytized by amoebas are unknown. However, based on transmission electron microscopy and biological assays, purified giant vesicles are indeed infectious.[17] Single (isolated) particles can be found inside amoebal endosomes 10 minutes after inoculation.[50] Twenty minutes postinfection, a progressive alteration in particle appearance is observed, including degradation of the protein shell. The virion core is then transferred to the cytoplasm through an opening in the endosome membrane after interaction with the inner membrane of the virus particle (Fig. 17.6).[50]

Immunofluorescence and protein-labeled assays revealed that marseillevirus induces the migration of nucleolar and nuclear proteins to the cytoplasm at very early times of infection (30-60 minutes).[50] Because proteomic analysis of marseillevirus particles demonstrated the lack of transcriptional machinery elements in the virion, it was hypothesized that such nuclear elements are recruited to the cytoplasm to promote the expression of marseillevirus immediately early genes. Interestingly, between 2 and 4 hpi, nuclear and nucleolar proteins return to the nucleus (Fig. 17.6).[50]

The electron-lucent viral factory occupies one-third to one-half of the amoebal cytoplasm. The morphogenesis of particles usually starts in the central region of the virus factory, from crescentlike 50-nm structures.[17,34] Dense fibrillar elements are incorporated by forming particles, suggesting that capsid shell synthesis and core element (genome/proteins) incorporation occur simultaneously. However, this point of the cycle requires further investigation. From 5 hpi, the viral progeny can be detected inside infected cells.[34]

After morphogenesis, mature particles migrate to the periphery of the viral factory. Adjacent to this region, a membrane-rich area is observed, where a large portion of the particles are wrapped, forming the aforementioned giant vesicles.[17] When those vesicles present only one membrane layer, they migrate to the cell periphery and fuse with the plasma membrane, releasing free particles to the outside environment. When they present two or more membranes, they fuse the outer one with the cell plasma membrane, releasing to the outside environment giant vesicles containing from a few to thousands of marseillevirus particles. Treating cells with brefeldin A affects both particle and giant vesicle

formation, indicating that membrane traffic is important to the marseillevirus replication cycle. Although the endoplasmic reticulum protein GRP78 has been found in purified vesicles, additional studies are necessary to reveal the origin of membranes present in those giant vesicles. After cell lysis (starting at 6 hpi), particles and giant vesicles are released (Fig. 17.6).[17]

Pandoraviruses

The first pandoraviruses described in 2013 were isolated from the Tunquen River at the central coast of Chile (*Pandoravirus dulcis*) and from a freshwater pond near Melbourne, Australia (*Pandoravirus salinus*).[119] Interestingly, in 2008, biological entities with morphological properties similar to pandoraviruses had been described, but they were defined as amoebal endocytobionts.[142] Inside their oval virions measuring 1 μm in length, pandoraviruses package linear dsDNA genomes that reach up to 2.77 Mb and represent the largest virus genomes described so far (*P salinus*) (Fig. 17.1).[119] In subsequent years, many pandoraviruses have been described, isolated from different substrates and continents, including *P massiliensis*, *P braziliensis*, *P kadiweu*, *P neocaledonia*, *P japonicus*, and others.[7,71,85,88,118] Comprehensive genomic, transcriptome, and proteomic studies have substantially accelerated the understanding of the biology of this fascinating group of viruses.

Virion Structure

Pandoravirus particles are oval and measure about 1 μm in length and 0.5 μm in width, with a predicted volume of 7.5×10^7 nm^3 [119] (Figs. 17.1 and 17.7). The particle has a 70-nm-thick tegument, composed of three layers (20, 25, and 25 nm, from inside to outside), with the middle layer being the most electron dense. There is an internal lipid membrane juxtaposed to the tegument, which in turn surrounds an electron-lucent region containing the genome and proteins. A spherical structure, about 50 nm, has been described at varying positions in the particle's core. At one end of the particle, the tegument opens into an ostiole (~90 nm in diameter). The genome is released from this ostiole in the uncoating phase.

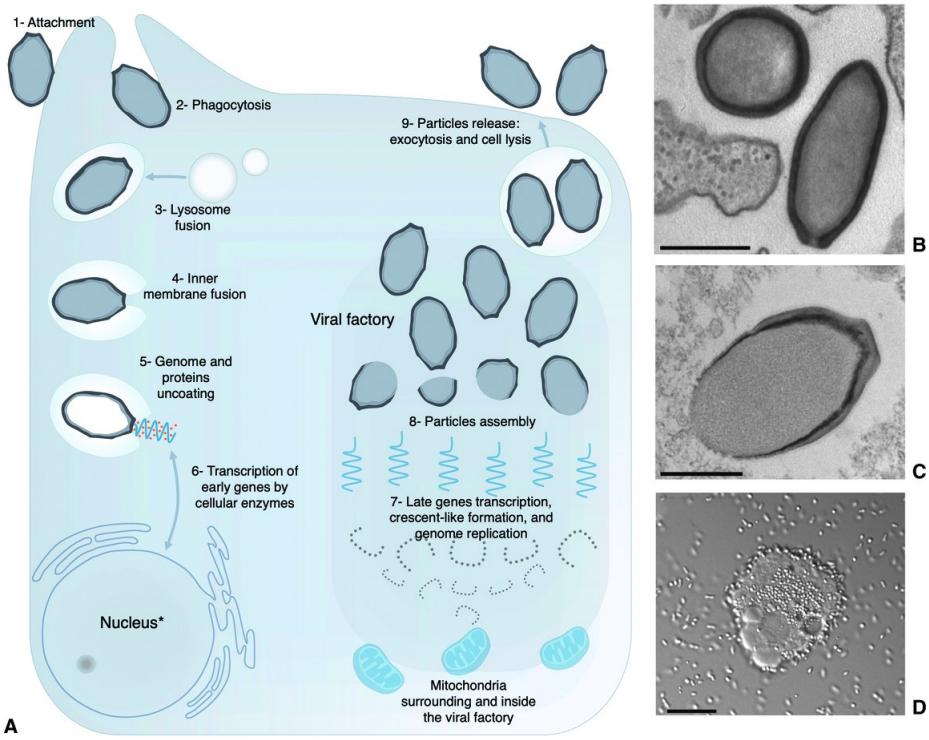


FIGURE 17.7 **Pandoravirus replication cycle and structural features**. **A**. Schematic overview of the pandoravirus replication cycle. 1 and 2—Pandoravirus particle attachment to the cell membrane induces pseudopod formation and phagocytosis. 3-5—Lysosome fusion with phagosomes induces the pandoravirus inner membrane fusion with the phagolysosome, releasing the virus genome and proteins into the host cytoplasm. 6—Cellular transcriptional enzymes promote the expression of pandoravirus early genes. 7—Upon the formation of the viral factory, mitochondria and membranes are recruited to the factory. Late genes are expressed in the viral factory, followed by crescent formation and virus genome replication. 8—Particles are assembled, concomitantly incorporating the genome and structural elements. 9—Particles are wrapped into large vesicles and then exocytosed. Eventually, the host cell lyses, releasing the rest of the pandoravirus particles. *During the late phase of pandoravirus infection in *Acanthamoeba castellanii*, the nucleus and the nucleolus are no longer observed. **B**. Transmission electron microscopy of an ultrathin section of pandoravirus particles (*P neocaledonia*). Scale bar: 500 nm. **C**. Transmission electron microscopy of an ultrathin section of a nascent pandoravirus (*P quercus*) particle. Scale bar: 500 nm. **D**.*A castellanii* infected by pandoravirus (*P macleodensis*), observed by optical light microscopy. Pandoravirus particles can be visualized inside and outside the cell, with their typical oval-shape. Scale bar: 10 μm. (Images in panels **B**-**D** are adapted from Legendre M, Fabre E, Poirot O, et al. Diversity and evolution of the emerging Pandoraviridae family. *Nat Commun*. 2018;9(1):2285. http://creativecommons.org/licenses/by/4.0/.)

P massiliensis particles may have a membrane potential (proton gradient).[8] This result has been observed for purified virions and particles in the context of the replication cycle. About 20% of the observed particles were concomitantly labeled by anti-pandoravirus antibodies (FITC conjugated) and by MitoTracker. In addition, purified particles were also stained with tetramethylrhodamine methyl ester, a molecule acknowledged as a marker to assess mitochondrial membrane potential, further supporting the membrane potential of these particles. Treatment of *P massiliensis* virions with the depolarizing agent carbonyl cyanide chlorophenylhydrazone abolished the potential membrane of

the particles. The role of this possible proton gradient in energy generation during the pandoravirus cycle remains to be investigated.[8]

The treatment of *P massiliensis* with calcofluor indicated that cellulose might be present in the viral tegument wall. Incubation of the particles with cellulase caused degradation of the tegument. However, *P massiliensis* lacks genes clearly associated with cellulose synthesis. It has been hypothesized that the cellulose present in the viral tegument is synthesized by the amoebal machinery, because the amoebal cellulose synthetase gene appears to be expressed during the phase of viral morphogenesis.[35]

An in-depth study of virus particle proteomics, using high-sensitivity methods, revealed wide variation in the number of proteins detected in different pandoravirus isolates: 424 for *P salinus*, 357 for *P quercus*, 387 for *P dulcis*, and 337 for *P neocaledonia*. However, it was assumed that most of the detected proteins could be contamination from host proteins, randomly incorporated or externally associated with the particles.[88] A more stringent analysis revealed that a total of 102 protein clusters are shared by all four pandoravirus strain particles analyzed (core proteome). Interestingly, the genes encoding the core proteome present the strongest purifying selection among all pandoravirus genes.[88] Only 19 out of 102 clusters of the core proteome could be associated with identifiable functional and structural motifs. Four of these 19 proteins exhibit generic motifs, including two with a collagenlike domain, one with a Pan-APPLE–like domain, and 1 with a terminlike domain.[88]

Some proteins involved in redox processes were detected in particle proteomics, including an ER-like thiol oxidoreductase, a protein with slight similarity to a thioredoxin reductase, a putative NAD-dependent amine oxidase, and a FAD-coupled dehydrogenase. Ser/Thr kinase and phosphatase, a DNA-binding protein (probably related to genome packing) and endoribonuclease domain proteins were also detected. An MCP-like protein was detected not in pandoravirus particles, nor were other nucleocytoviricota hallmark proteins, such as DNA-packing ATPase and DNA-binding core protein.[88] Multiple sequence alignments and remote homology searches indicated that the second most abundant protein present in the pandoravirus proteome evolved from an inactivated derivative of a bacterial glycoside hydrolase of family 16 (GH16).[79] RNA polymerase was not found in the particle proteomes, suggesting that the early stages of the pandoravirus cycle require the host's transcription machinery located in the nucleus.[88] Similar results were described for the *P massiliensis* particle proteome, although 162 proteins were found in the virion, including 99 hypothetical proteins.[8] Therefore, pandoravirus particles are mainly made up of proteins with no known homologues.

Genome Structure and Organization

Pandoravirus genomes are linear dsDNA molecules with a GC content of about 61%. A wide variation in the size of the genomes of different viral isolates has been observed, ranging from 1.6 Mb in *P massiliensis* to 2.5 Mb in *P salinus*.[7,88] There is evidence that the *P salinus* genome can reach up to 2.77 Mb due to variations in terminal tandem repeats.[119] Therefore, pandoravirus genomes are the largest described in the known virosphere (Fig. 17.1). Initial predictions of the number of genes present in the genomes of *P dulcis* and *P salinus* revealed 1502 and 2556 putative CDS, respectively, corresponding to about 90% of ORFans.[119] However, a combined genomic and RNA-seq analysis of a set of pandoravirus isolates revealed that these initial numbers of CDS had been overestimated, especially with regard to ORFs < 300 nucleotides. In light of this analysis, a total of 1430 ORFs were predicted for *P salinus*.[88] This impressive reduction in the number of predicted ORFs seems to be the result of the combination of a high GC content and a large number of ORFans. Hence, a combined genomic and RNA-seq approach is necessary to obtain greater accuracy in predicting bona fine ORFs. Even with these more stringent parameters, up to 73% of pandoravirus ORFs are ORFans.[88]

In addition to conventional transcripts, a number of long noncoding transcripts (lncRNAs) were detected: up to 268 in *P dulcis*. These lncRNAs have a poly(A) tail, and 4% of them have spliceosomal introns and probably regulate pandoravirus gene expression. Overall, up to 87% of the pandoravirus genome appears to be transcribed, but only 68% appears to be translated into proteins.[88]

As observed for most nucleocytovirus families, the analysis of the genomes of several pandoravirus isolates has revealed an open pangenome; therefore, the discovery of new isolates continues to increase knowledge of new groups of orthologous genes.[8,88] Analysis of the organization of pandoravirus genomes has shown a collinearity of homologous blocks of about 80%. Interestingly, one-half of the genome is more conserved, carrying a good part of the genes of the core genome, thus suggesting that they are essential genes. The other half of the genome is highly variable, with more insertions and inversions of syntenic blocks.[88] Researchers have observed abundant tandem gene duplication.[88] In addition, based on G + C content, ORF length, and CAI analyses of clade-specific or core genes, it has been proposed the eukaryoticlike *de novo* gene creation hypothesis for pandoraviruses genomes.[88]

A large proportion of the few ORFs with detectable homologs in databases putatively encode proteins containing motifs and domains like ankyrin, MORN, and F-box; this limited homology does not allow reliable speculations about their biological functions. However, the identification of 14 genes commonly shared by other large and giant DNA viruses reinforces the classification of pandoraviruses among members of the phylum *Nucleocytoviricota* (Fig. 17.1). Among the identified genes, a DNA polymerase, a virion-packing adenosine-triphosphatase, an mRNA-capping enzyme, and subunits of the DNA-dependent RNA polymerase are noteworthy.[7,71,88,119] A substantial number of genes involved in DNA processing and transcription have also been identified in the pandoravirus genome, in addition to several components of the ubiquitin-dependent protein degradation pathway. Translation-associated factors have also been found, although in a smaller number than those observed for *Mimiviridae*: two aaRS (Tyr and Trp), three tRNAs, and one translation initiation factor (eIF4E). Genes encoding DNA ligases, topoisomerases, and a DJR-like major capsid protein have not been identified in pandoravirus genomes.[119]

Stages of Replication

Several studies have described *A castellanii* as a known host of pandoraviruses.[7,71,118,119] Transmission and scanning electron microscopy have shown that pandoravirus particles can induce pseudopod formation in host cells.[118] The particles are internalized, one by one, probably by phagocytosis (Fig. 17.7). Several particles can be internalized by a single cell. Such particle-containing phagosomes appear to fuse with lysosomelike structures, and it is speculated that acidification of the phagosome results in uncoating.[118] During this process, in the ostiole region, the inner membrane of the particle fuses with the membrane of the phagosome, releasing the genome and viral proteins into the cytoplasm.[118,119] Between 2 and 4 hpi, the cell nucleus begins to show morphological changes by losing its rounded shape. The nucleolus gradually becomes electron-lucent in electron microscopy images, until it is no longer visible.[118,119] Although such modifications have been observed for most pandoraviruses, some isolates show temporal differences in relation to the induction of the described sequence of events.[119]

The pandoravirus factory is visualized by electron microscopy as a large >2-μm electron-lucent structure (Fig. 17.7). This factory can occupy about one-third of the host cell. Recruitment of mitochondria and membranous structures into the viral factory is observed from the beginning of factory formation until the morphogenesis of the first particles.[118,119] Due to the absence of the transcription machinery in pandoravirus virions, it is very likely that elements of the cell nucleus participate in the transcription of viral immediately early genes. However, studies are still needed to confirm whether these elements are recruited to the cytoplasm, as described for the marseilleviruses.[50,88]

The morphogenesis of pandoravirus particles seems to start with the formation of semicircular electron-dense structures, measuring about 190 nm, in the center of the viral factory. Such structures become thicker and electron-dense with the incorporation of more structural layers, assuming a crescentlike appearance. The tegument development and the filling of the particle with genomic material, replicated in the cytoplasm, occur concomitantly, mainly starting from the ostiole region. The formation of the first mature particles occurs, on average, from 6 hpi onward.[118,119] Cytopathic effects such as rounding, circular movements, and formation of intercellular bridges have been described for cells infected with pandoravirus.[62]

As mature particles are formed, long membranous structures become evident in the viral factory. Such membranes wrap particles on the periphery of the factory, forming vesicles of varying sizes, containing from one to nearly a dozen particles. The vesicles can be seen from 6 to 8 hpi, depending on the pandoravirus isolate, and probably correspond to exosomes. From this moment on, it is possible to observe many of these structures in the periphery of host cells, and an increasing accumulation of virus particles in the extracellular environment, without cell lysis occurring so far (Fig. 17.7). This observation suggests that the release of particles occurs by exocytosis.[118,119] The treatment of infected amoebas with brefeldin A negatively impacts the transport of virus particles to the extracellular environment.[118] In the later stages of the replication cycle, virus particles are visualized in different regions of the cell, and the nucleus is completely degraded, as observed for most known pandoravirus isolates. Cell lysis eventually takes place, releasing the rest of the virus particles.[118,119]

"Extended *Asfarviridae*": Faustovirus, Kaumoebavirus, and Pacmanvirus

Virus isolation studies using *V vermiformis* as a host resulted in the discovery of faustovirus in 2016 and of kaumoebavirus in 2018.[21,126] This new group of viruses, also known as "extended *Asfarviridae*" phylogenetically clusters with the animal-infecting African swine fever virus (family *Asfarviridae*), and also comprises pacmanvirus, isolated from *A castellanii*.[14] The members of "extended *Asfarviridae*" of protists are dsDNA, linear or circular, ranging from ~350 kb (kaumoebavirus) to ~490 kb (faustovirus), with genomes substantially larger than those found in some strains of African swine fever viruses (~170 kb) (Fig. 17.1). Although the studies on "extended *Asfarviridae*" are still in the initial phase, recent information has revealed that some members possess unique structural and genomic characteristics.

Virion Structure

Among the "extended *Asfarviridae*," the faustovirus virions have been studied the most, by using MCP crystallization methods and cryo-EM. Faustovirus particles have an outer and an inner protein capsid shell, which is rare among large icosahedral dsDNA viruses.[76] The faustovirus MCP (645 amino acids) forms a homotrimer with each monomer consisting of two sequential jelly-rolls. MCP capsomers in the particle are arranged in an icosahedral fashion with a triangulation number of 277 (h = 7; k = 12). The inner shell has T = 16 (h = 4; k = 0) quasi-icosahedral symmetry and varies in diameter from 160 nm (without DNA) to 190 nm (filled with DNA). The whole particle, including the inner and outer shells, can reach up to 260 nm in diameter (Fig. 17.8). Cement proteins are observed under the capsid protein and presumably serve to maintain the stability of the outer shell.[76] Fifty angstroms-long, thick fibers decorate the particle surface, especially at the fivefold vertices. Although small amounts of lipids have been detected in faustovirus particles, it was hypothesized that the inner membrane has been replaced by the inner protein shell. Further analysis could confirm whether such detected residual lipids represent sample contamination. Faustovirus particle proteomics revealed 162 proteins, 76 of which had homologs in GenBank including MCP and A32-like packing ATPase.[76,126]

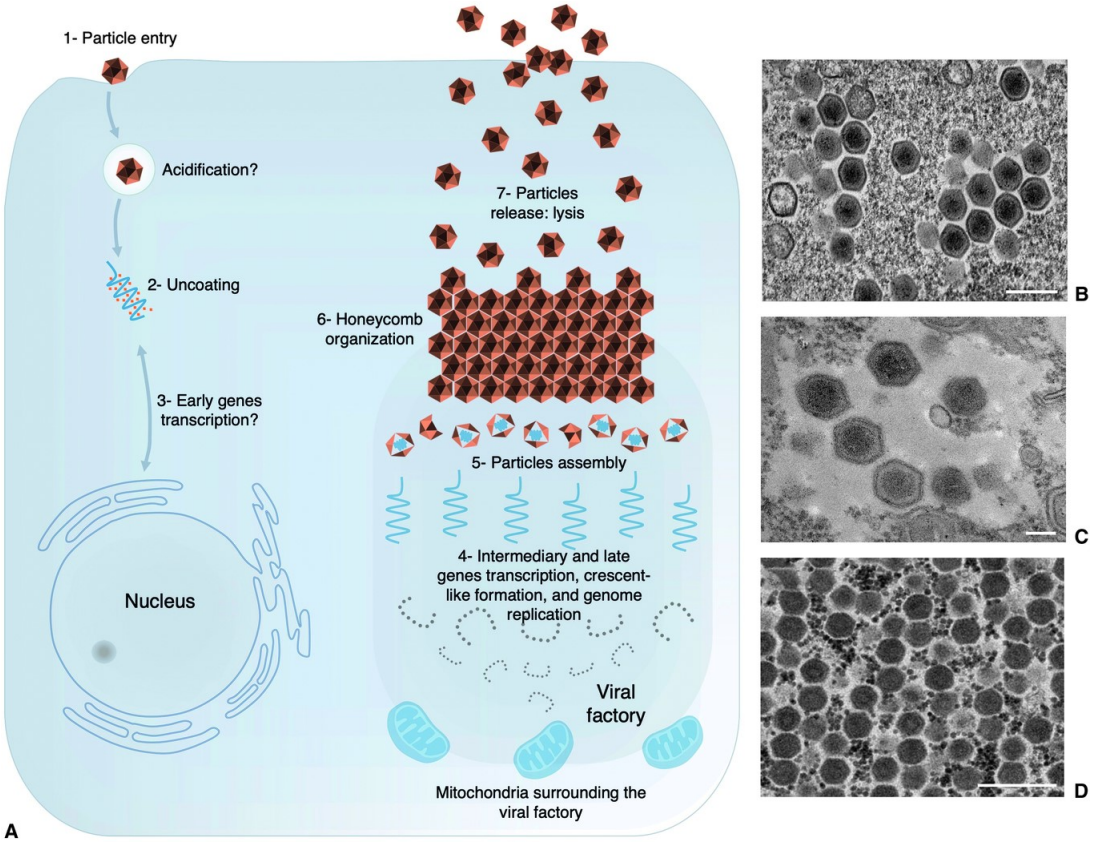


FIGURE 17.8 **Replication cycle and structural features of viruses of the extended *Asfarviridae***. **A**. Schematic overview of the faustovirus replication cycle. 1-3—Particles enter the amoeba by unknown mechanisms. The virus genome and proteins are released into the host cytoplasm, and it is believed that transcription of early genes is promoted by nuclear machinery. 4 and 5—Intermediate and late genes are expressed, and a large viral factory is formed in the cytoplasm. The virus genome is replicated, and particle morphogenesis starts from crescentlike structures. The genome is incorporated into particles. 6 and 7—Newly formed particles are organized in a honeycomb fashion and eventually are released after cell lysis. **B**. Transmission electron microscopy of faustovirus (*F mariensis*). Scale bar: 200 nm. (Courtesy of Iara Borges and Jônatas S. Abrahão, Center of Microscopy, Universidade Federal de Minas Gerais, Brazil.) **C**. Transmission electron microscopy of a kaumoebavirus isolate. Scale bar: 100 nm. (Courtesy of Victória Queiroz and Jônatas S. Abrahão, Center of Microscopy, Universidade Federal de Minas Gerais, Brazil.) **D**. Transmission electron microscopy of a pacmanvirus. Scale bar: 500 nm. (Courtesy of Julien Andréani and Bernard La Scola, Aix-Marseille Université, France.)

For pacmanvirus, cryo-EM analyses showed icosahedral particles with a spiky outer surface and an inner membrane. Unlike faustovirus, no inner protein shell was observed.[14] Pacmanvirus particles are about 250 nm, with a triangulation number of 309 (h = 7; k = 13). Its MCP has a typical double jelly-roll fold. The protein PACV_341 was identified at the icosahedral fivefold axis as the putative minor capsid protein.[14] For kaumoebavirus, future structural and proteomics studies of its 250-nm-wide virions may elucidate whether this virus share characteristics with other members of the group.

Genome Structure and Organization

More than a dozen faustovirus strains have been isolated from samples collected in France, Senegal, Lebanon, and Brazil.[25,31,126] Sequencing the genomes of some of these strains has enabled comparative analyses on their evolution, gene conservation, synteny, and specific features. On average, the faustovirus genomes are ~470 kb long, with a G/C content of about 37%. At least four faustovirus lineages have been identified based on core-gene analysis.[25] Regarding faustovirus E12, the first to be described, a total of 457 genes were predicted, including more than 300 ORFans. Paralogs represent about one-fifth of the genes.[126] A number of genes shared by other protist-infecting viruses have been predicted in faustovirus, kaumoebavirus, and/or pacmanvirus, including some related to DNA replication, recombination, and repair; transcription; and RNA processing.[14,21,25,126] However, translation-related elements are rare in this group (a single tRNA, for Ile, was predicted in pacmanvirus).[14] The motifs TATTT and TATA are abundant in intergenic regions of faustoviruses and kaumoebavirus—as has been described for asfarvirus—suggesting that those motifs may act as gene promoters.[114]

It is noteworthy that the MCP-encoding gene has an atypical arrangement in faustovirus genomes, stretching over 17 000 bp with up to 18 introns (including group I and spliceosomelike introns). Splicing results in a 1939 bp transcript that encodes a 645 amino acid protein.[25,37,126] Similarly, the kaumoebavirus MCP gene spans a 5000 bp region (nucleotides 180 327-185 966), including at least three distinct exons.[22,66] Although faustovirus genomes exhibit a highly conserved synteny, their terminal regions and the central region containing the MCP gene have evolved at faster rates compared with other genomic regions, containing more indels and rearrangements.[67] On the other hand, the ~395 kb pacmanvirus encodes a single MCP ORF—as has been described for asfarvirus—indicating that the colonization of faustovirus and kaumoebavirus MCP genes by introns occurred after diversification of this group.[14] Pacmanvirus has three copies of genes encoding proteins containing PAN/apple domains and a tRNA His guanylyltransferase, which is not found in asfarvirus, kaumoebavirus, and faustovirus.[14]

Stages of Replication

Vermamoeba vermiformis is the only known host for faustovirus and kaumoebavirus.[21,126] In contrast, pacmanvirus infects *A castellanii*.[14] Faustovirus and kaumoebavirus cause trophozoite lysis around 18-20 hpi.[21,126] On the other hand, the pacmanvirus cycle is remarkably rapid, causing cell lysis starting at 6 hpi.[14] A common feature among faustovirus, kaumoebavirus, and pacmanvirus is that internalization of the particles takes place between 30 minutes and 3 hours, individually or in particulate clumps, by a still unidentified endocytic or phagocytic routes hpi.[14,21,126] In early stages, transmission electron microscopy revealed particles in regions adjacent to mitochondria (pacmanvirus) or even within the cell nucleus (faustovirus).[14,31] However, most virions are observed in free endosomes/phagosomes in the cytoplasm in the early stages of the cycle, indicating that virion uncoating occurs at this site. In the early stages of faustovirus infection, the cell nucleus undergoes morphological modifications, becoming more elongated and with the nucleolus in a more peripheral position.[31,126] From 3 to 6 hpi, signal transduction regulation transcripts are highly expressed by faustovirus, indicating a possible modulation of cell pathways that precedes viral factory formation.[37]

The viral factories of faustovirus are electron-lucent structures of more than 3.5 μm in diameter. A large number of mitochondria migrate to the periphery of faustovirus factories, suggesting virus-induced energy optimization during viral morphogenesis (Fig. 17.8).[31,126] From 6 hpi onward, expression of transcripts related to structural proteins increases.[37] The morphogenesis of faustovirus starts from 50 nm crescentlike structures, which gain in complexity until they form empty immature particles with a small opening. The particle is then filled by the virus genome and proteins. Then, particle closure occurs, presenting an electron-dense core.[31] As the mature virions are formed, they are organized in a honeycomblike fashion as geometrically arranged clusters (Fig. 17.8).[14,31,126]

Virus particle release occurs individually after plasma membrane rupture, or in large clusters of particles,[14,31,126] or alternatively for kaumoebavirus, release by exocytosis has been suggested.[21]

It is noteworthy that a unique antiviral mechanism has been described in *V vermiformis* upon faustovirus infection. Once infected by this virus, some *V vermiformis* trophozoites release a soluble factor, possibly containing Mg^{2+}. This soluble factor induces neighboring cells to turn into cysts. If these cells are infected, the virus cycle is aborted and virus particles are stably imprisoned in nonviable cysts, reducing the amount of viral progeny in the supernatant and impacting the overall infection rate of the amoeba's population. Noninfected cells also encyst upon contact with this soluble factor, and eventually excyst and survive.[31] This mechanism illustrates how the discovery and study of new giant viruses can reveal novel biological processes.

Pithovirus, Cedratvirus, and Orpheovirus

The search for new giant amoeba viruses has inspired research into diverse geographical locations. Pithovirus sibericum was described in 2014, isolated from samples collected from Siberian permafrost in Russia (Kolyma lowland region).[87] Radiocarbon dating indicated that this substrate corresponded to the late Pleistocene, ~30 000 years old. This virus was isolated after inoculation of the aforementioned substrate in cultures of *A castellanii*, causing cytopathic effects similar to those observed for other giant viruses. Pithovirus sibericum particles resemble pandoraviruses in their oval appearance; however, further analysis showed that pithovirus virions are ~1.5 μm long, being, on average, the largest virus particles described so far, although their genome is only 600 kb (Fig. 17.1).[87,119] In subsequent years, other pithoviruslike viruses have been discovered, including a new group called cedratvirus,[12] also isolated in *A castellanii*, as well as orpheoviruses, capable of infecting *V vermiformis*.[13]

Virion Structure

Pithovirus particles are oval and have a mean length of 1.530 ± 230 nm and a mean diameter of 810 ± 60 nm (Fig. 17.9)[87,112]; however, some can reach up to 2.5 μm in length and 0.9 μm in diameter.[112] Cryo-EM analyses also revealed that the particles have a wall ~60 nm wide formed by a structure called the tegument, which is organized in parallel bands. The tegument contains a poorly characterized structure called the low-density out-layer, and it was speculated that such a layer is composed of materials such as polysaccharides.[87,112] Below the tegument is an inner membrane surrounding the so-called nucleoid, a region of the virion that houses the genome and other elements (early-stage proteins). At one end of the virion is an apical pore sealed by a cork, 80 nm thick and 160 nm wide.[87,112] The cork appears to be composed of 15-nm-wide parallel stripes that resemble a honeycomb when viewed from above. Energy-filtered cryo-EM at 200 kV revealed an additional horizontal Moiré pattern on the stripes in the side view.[112] Although most pithovirus virions contain only one cork, particles containing two corks, nonaligned at both ends of the particle, similar to cedratviruses, have also been observed less frequently.[112] The mean volume of pithovirus particles is 26.2 ± 5.1 × 10^7 nm^3. The density of the nucleoid region of pithovirus sibericum particles is 0.77 times less than that observed in mimiviruses.[112]

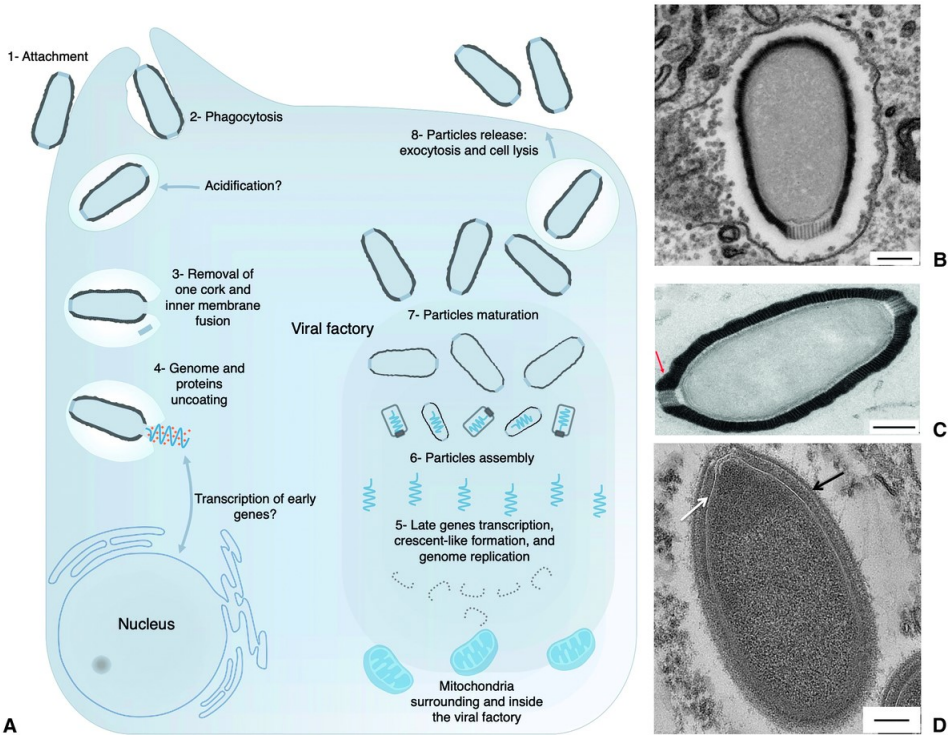


FIGURE 17.9 **Replication cycle and structural features of pithoviruses**. **A**. Schematic overview of the cedratvirus replication cycle. 1 and 2—Cedratvirus particle attachment to the cell membrane induces pseudopod formation and phagocytosis. 3—It is not known whether phagosome acidification is required for the removal of one cork and viral inner membrane fusion with the phagosome. 4—The virus genome and proteins are released into the host cytoplasm. 5 and 6—In the viral factory, genome replication, transcription of late genes, and particle morphogenesis occur. The assembly of particles is complex and sequential, involving intermediates with diverse shapes. 7 and 8—The particles undergo maturation, including tegument thickening. Then, particles are released by exocytosis and cell lysis. **B**. Transmission electron microscopy of a pithovirus (*P sibericum*) particle. Scale bar: 200 nm. (Courtesy of Chantal Abergel, CNRS, Aix-Marseille Université, Information génomique & structurale (IGS), UMR 7256, Institut de microbiologie de la Médit4erranée (IMM), France.) **C**. Transmission electron microscopy of a cedratvirus (*C getuliensis*) particle. Scale bar: 200 nm. The *red arrow* indicates the cork region. (Adapted from Silva LKDS, Andrade ACDSP, Dornas FP, et al. Cedratvirus getuliensis replication cycle: an in-depth morphological analysis. *Sci Rep*. 2018;8(1):4000. http://creativecommons.org/licenses/by/4.0/.) **D**. Transmission electron microscopy of an orpheovirus IHUMI-LCC particle. Scale bar: 100 nm. The *black arrow* and *white arrow* indicate the integument and the inner membrane, respectively. (Adapted from Andreani J, Khalil JYB, Baptiste E, et al. Orpheovirus IHUMI-LCC2: a new virus among the giant viruses. *Front Microbiol*. 2018;8:2643. http://creativecommons.org/licenses/by/4.0/.)

Proteomic analysis of pithovirus particles identified 159 different gene products, two-thirds of which correspond to proteins with unknown functions.[87] Pithovirus particles have all the transcription machinery proteins encoded by their genome. Proteins such as an adenylosuccinate synthetase (pv90), an ADP-ribosyl glycohydrolase, protein kinases (pv156, pv405, and pv438), hydrolases (pv212 and pv289), oxidoreductases (pv342 and pv467), and a dehydratase (apv385) were also found. No proteins with clear homology to MCP or major core protein were detected and, therefore, the elements that compose the tegument remain to be investigated. The four most abundant proteins detected by proteomics do not correspond to proteins with known homologs (pv449, pv461, pv93, and pv106). Finally, 37 proteins from *A castellanii* were detected in low abundance in pithovirus particles.[87]

Characterization of the first cedratviruses in 2016 revealed 1.2-µm-long virions with a shape very similar to those of pithoviruses, including regions such as the tegument, gap, inner membrane, and nucleoid region.[12] However, the most striking feature of cedratviruses is the presence of two cork-covered ostioles in most particles (Fig. 17.9). Different cedratvirus isolates show variations in their particle sizes, as highlighted for the Brazilian cedratvirus strain, whose particles have an average size of 910 nm in length and 510 nm in width.[128]

Orpheoviruses—despite being phylogenetically related to pithoviruses and cedratviruses—present particles with substantial structural differences in relation to the rest of the known pithoviruslike viruses.[13,155] Orpheovirus particles are oval, with average dimensions of up to 1300 nm along its longer axis (Fig. 17.9). Externally, 30-nm-long fibril-like structures cover a tegument formed by two layers each about 5-10 nm wide. Unlike pithovirus and cedratvirus teguments, the orpheovirus tegument appears electron-lucent when viewed with transmission electron microscopy.[13,155] A membrane inside the two tegument layers encloses the genome and proteins (nucleoid).[13,155] A lateral flattening of the mature particles has been described.[155] Corks covering the ostiole have not been observed in orpheoviruses, and the elements that protect this opening have not yet been characterized.[13,155] Future proteomic studies for cedratviruses and orpheoviruses may reveal shared similarities among members of the pithoviruslike group.

Genome Structure and Organization

Despite having gigantic virus particles (on average, 1.5 µm long), the genome of pithovirus sibericum is a 610 033 bp dsDNA molecule, considerably smaller than that observed for other giant viruses, such as mimiviruses and pandoraviruses (Fig. 17.1).[87,119,124] The genome is either circular or a terminally redundant circularly permutated linear DNA molecule, with a high repeat content, and is predicted to encode 467 proteins. At the time of its genome description, 32.5% (152/467) of the proteins had recognizable homologs. Of these proteins with recognizable

homology, the best matches are distributed similarly between eukaryotes, bacteria, and viruses.[87] The low percentage of similarity between pithovirus genes and their best matches suggests that Pithovirus sibericum is the first representative of a new virus family. Of the 152 proteins predicted with homologs in databases, only 125 have functional attributes, including genes related to DNA transcription, DNA repair, nucleotide synthesis, DNA replication, carbohydrate processing, RNA processing, hydrolases, and oxidoreductases.[87]

Unlike other giant amoeba viruses such as mimiviruses, no component of the protein translation machinery has been found in the genome of pithovirus.[87] The gene encoding the DNA-dependent RNA polymerase large subunit has a group I self-splicing intron. Interestingly, a low similarity (21% of identical residues, e-value = 0.002) was observed between the predicted protein product for the pv460 gene and the divergent MCP of iridoviruses (megalocytiviruses). Bioinformatic analyses suggested that pv460 assumes the jelly-roll type structure, common among viruses belonging to the phylum *Bamfordvirae* (Fig. 17.2). However, pv460 was not detected in virus particle proteomics.[87] Although the 589 068 bp genome of cedratvirus A11 presents great similarity with the main aspects described for the pithovirus, 30.8% of its predicted proteins (177/574) did not present matches with the databases, therefore being considered ORFans.[12]

With a dsDNA genome of 1 473 573 bp, orpheovirus IHUMI-LLC2 has the largest genome described so far among the pithoviruslike viruses (Fig. 17.1).[13] A total of 1199 genes were predicted, with 690 (~57.5%) being considered ORFans. The best hit profile observed for orpheovirus predicted genes follows a similar distribution described for other pithoviruslike viruses, matching with eukaryotes, prokaryotes, and viruses. However, similarly to mimiviruses, orpheovirus has an extensive arsenal of genes associated with protein translation, including eight aaRS and four translation factors (three initiation factors and one release factor).[3,15,22]

Stages of Replication

Current data indicate that pithoviruses and cedratviruses replicate in *A castellanii*, whereas orpheoviruses are capable of infecting *V vermiformis*.[12,13,87,153,155] The pre-entry stages are still poorly characterized, but it was demonstrated that virus particles for the isolate Cedratvirus getuliensis are able to adhere to the surface of *A castellanii* cells, suggesting the involvement of possible adhesion factors (Fig. 17.9).[153] Because the virions of pithovirus, orpheovirus, and cedratvirus are >500 nm, it is assumed that they are able to trigger phagocytosis in amoebas.[175] In fact, considering all these viruses, there is an abundance of evidence to support this hypothesis. The induction of pseudopod formation has been described after inoculation of *V vermiformis* with orpheovirus.[155] Assays using inhibitors of endocytic and phagocytic pathways have shown that the treatment of amoebas with cytochalasin affects the uptake of cedratvirus particles, while treatment with EIPA or chloroquine does not have such a reducing effect, reinforcing that phagocytosis might be important to orpheovirus entry.[153] After phagocytosis, pithoviruslike virions undergo structural changes that induce the exposure of the internal membrane of the particles. In the case of pithoviruses, the cork is detached from the virion, and then the inner membrane fuses with the membrane of the phagosome, forming a channel that projects the internal content of the particle into the cytoplasm of the host amoeba.[87] The cedratviruses, as mentioned previously, have two corks, but electron microscopy analysis indicates that only one of the corks is removed preceding the genome uncoating (Fig. 17.9).[12,153] In the case of the cork-less virions of orpheovirus, the inner membrane is projected from the ostiole localized at the apex of the particle, also fusing with the phagosome membrane, and then releasing the genome and proteins into the host cytoplasm.[13,155]

The formation of the pithovirus, cedratvirus, and orpheovirus factories occurs in a region close to the cell nucleus, which in turn, remains morphologically intact throughout the replication cycle (Fig. 17.9).[12,13,87,155] The recruitment of mitochondria to the periphery of the viral factory has been described for cedratvirus and orpheovirus, indicating a possible energy optimization related to the formation of new particles.[153,155] The pithovirus, cedratvirus, and orpheovirus factories occupy a large portion of the amoeba's cytoplasm, comparable to the volume occupied by the cell nucleus. These factories show an electron-lucent appearance on electron microscopy, very different from the electron-dense factories of mimiviruses.[11–13,87,155,162]

The morphogenesis of pithovirus, cedratvirus, and orpheovirus virions involves a complex and sequential organization of immature viral structures, starting at the center and moving to the periphery of the viral factories. This process has been characterized extensively for infection with viruses corresponding to the isolate Cedratvirus getuliensis (Fig. 17.9), but it appears to be very similar to pithoviruses.[87,153] The first discernible structures during particle assembly are the crescent-shaped precursors (~100 nm), very similar to the crescents described for other nucleocytoviruses.[127,153] These crescents appear to grow lengthwise, reaching about 600 nm, assuming a staplelike shape. The cross section of these structures reveals a horseshoe-like shape, and at this stage, only one cork is visible (cedratvirus) in the longitudinal section, probably at the pole where the morphogenesis starts.[153] The immature particles appear as open cylinders: in the longitudinal section, they appear as rectangles, and in the cross section, they appear as circles. Then, a progressive filling of the capsid is observed, followed by its closure and then the addition of the second cork (in the case of cedratviruses).[153] After closing the structure, the particle undergoes differentiation processes in a region very close to the periphery of the viral factory, resulting in thickening of the tegument.[87,153] At this stage, the tegument does not have the same thickness as the cork, but during maturation, the tegument thickens, giving rise to mature particles (Fig. 17.9). Between 6 and 12 hpi, immature and already formed particles coexist in abundance in the amoebal cytoplasm.[153]

The morphogenesis of the orpheoviruses has many similarities to that described for the other pithoviruslike viruses. Electron-dense semicircular crescents develop, extend, and then are filled with material present in the factory, including the genome and proteins needed for early phase.[13,155] At this stage, lamellar structures are recruited that possibly associate externally with the tegument of the particles in formation. Finally, the particles are closed and assume their beanlike appearance, with a lateral flattening.[13,155] Amoebas infected by orpheovirus present increased motility, mainly at 12 hpi. The detection of infectious orpheovirus particles occurs from 12 hpi and reaches a plateau after 48 hpi.[155]

Concomitantly with the formation of mature particles, there is intense trafficking of intracellular membranes, resulting in packing one or more particles in exosomes. These filled vesicles migrate to the periphery of the amoebas, releasing the virions to the outside environment. This phenomenon has been described for pithovirus, cedratvirus, and orpheovirus (Fig. 17.9).[12,13,87,153,155] The exocytosis of pithoviruslike virus particles has also been demonstrated by quantifying the accumulation of virus particles in the supernatant of infected cell cultures, a phenomenon that precedes cell lysis. Immunofluorescence microscopy analysis suggests the polarization of exosomes containing orpheovirus particles at one end of elongated amoebas.[155] Treatment of infected cells with brefeldin A negatively affects the formation of such particle-containing exosomes. Some particles are also released after cell lysis, which occurs from 24 hpi and continues up to 48 hpi.[155]

Mollivirus

Following the search for new giant amoeba viruses, a new type of giant virus was isolated from the same permafrost sample from which the first pithovirus strain (Pithovirus sibericum) had been obtained.[87,89] The so-called mollivirus (Putative species Mollivirus sibericum) has almost spherical 600-nm virions and a linear dsDNA 650 kb genome (Figs. 17.1 and 17.10). Metagenomic analysis of the mentioned permafrost sample revealed the presence of reads of mollivirus, pithovirus, and *Acanthamoeba*, reinforcing that such biological entities were in fact present in this frozen soil dated at an age of 30 000 years.[89]

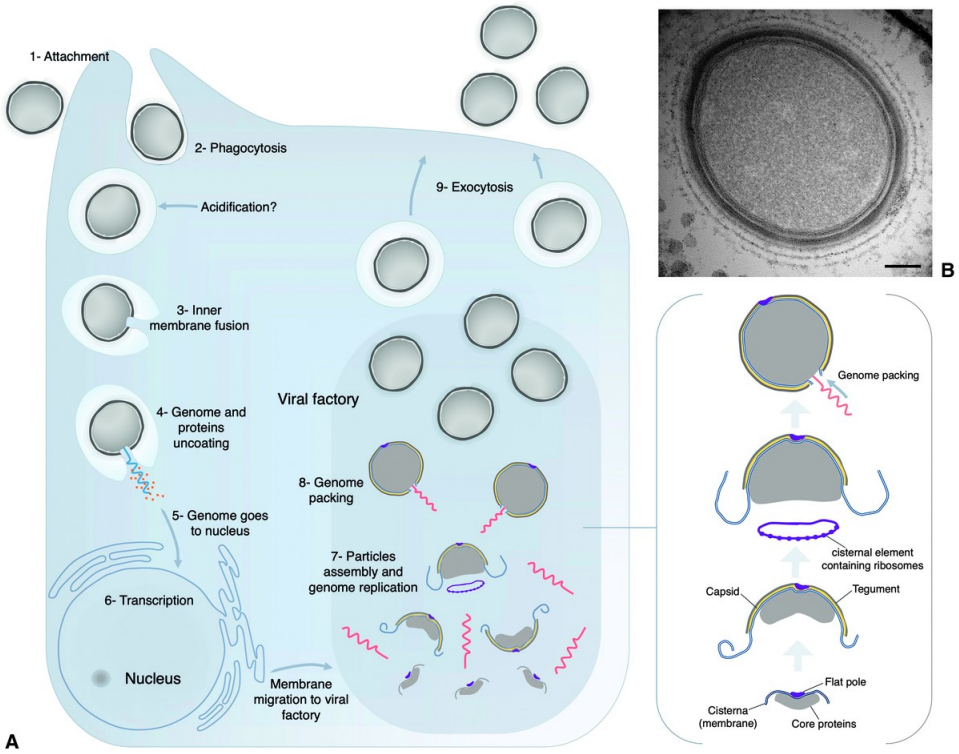


FIGURE 17.10 **Mollivirus replication cycle and structural features**. **A**. Schematic overview of the mollivirus replication cycle. 1 and 2—Mollivirus particles enter amoebas after attachment and phagocytosis. 3 and 4—The phagosome environment (likely acidified) induces mollivirus inner membrane fusion with the phagosome membrane, triggering the uncoating of virus genome and proteins into host cytoplasm. 5 and 6—The virus genome goes to the host nucleus, where its genes are transcribed. 7 and 8—Cellular membranes migrate to the newly formed viral factory and participate in particle morphogenesis. The sequential assembly and maturation of mollivirus particles are highlighted in the *bottom right panel*. Cisternalike membranes are associated with a flat pole and core proteins. Tegument and capsid layers are then incorporated into that structure, which expands with the addition of more membrane and core proteins. A cisternal element containing ribosomes contributes to the development of this structural complex. A mollivirus particle is then almost formed, keeping an open ostiole where the genome is incorporated. Finally, the ostiole is closed. **B**. Transmission electron microscopy of a mollivirus (*M sibericum*) particle. Scale bar: 100 nm. (Courtesy of Chantal Abergel, CNRS, Aix-Marseille Université, Information génomique & structurale (IGS), UMR 7256, Institut de microbiologie de la Méditerranée (IMM), France.)

Virion Structure

The ~600-nm-diameter mollivirus virions are almost spherical, surrounded by two to four ~25-nm spaced fibril rings (Fig. 17.10).[89,123] The outer fibril layers appear to be associated with the adjacent internal fibril layer. Alternatively, such a visual effect may be the result of the association of fibrils of different sizes relative to the tegument. In addition to fibril layers, the particles appear to be formed by two additional capsid layers and a tegument composed of two layers of fibrillar components, made of 30- to 40-nm interspaced parallel strips.[89,123] Inside the capsid layers, a membrane encloses the genome.[89,123] A circular fiber-free depression of about 200 nm is observed in the tegument. This region corresponds to the apex of the particle.[89]

Particle proteomics revealed 230 proteins, including 136 (59%) virus-encoded proteins and 94 (41%) corresponding to *Acanthamoeba*.[89] Among the proteins encoded by mollivirus, 74 (54.4%) are ORFans and only 35 (25.7%) of the viral proteins could be associated with functional or domain predictions. The three most abundant proteins in the particle are ORFans. A homologous protein of the MCP of other nucleocytoviruses, ML_347, was also detected in the virion, but despite its high abundance, it was not detected on the surface of the particle. In analogy to poxviruses, this protein could serve as a scaffold for the development of the tegument followed by proteolytic processing during the assembly, giving rise to nonicosahedral capsid.[89] Six of eight oxidoreductases predicted in the genome of mollivirus were detected in the virion. It has been speculated that such proteins may be important for particle resistance to the intraphagosomal environment of *Acanthamoeba*. None of the seven components of the predicted transcription machinery found in the genome of mollivirus was detected in the particle, which reinforces the dependence of the virus in relation to the *Acanthamoeba* nucleus at the beginning of the cycle (Fig. 17.10).[89]

The variety of amoebal proteins present in the virions of mollivirus is worth noting. Interestingly, several ribosomal proteins were consistently detected (S4, S7, S8, S9, S15, S23, L5, L6, L18, L30, and L35).[89] A total of five helicases, a translation initiation factor 4A, three enzymes involved in nuclear RNA processing and transportation, three histones, and HMG-like chromatin-associated proteins were also detected,

comprising a total of 13 different nuclear proteins carried in the virion. In addition, 11 proteins possibly involved in the transport of the virion to the nucleus and reorganization of the cytoskeleton were detected, including actin, propilins, and fascin.[89] Membrane prediction software analysis identified a total of 27 proteins containing at least one transmembrane domain, corresponding to about 20% of the proteome of the virions, including those involved in cell adhesion (ML_499), membrane fusion (ML_417), and lysosome-associated proteins (ML_309 and ML_331).[89,123]

Genome Structure and Organization

The genome of the Siberian mollivirus isolate consists of linear dsDNA of 651 523 bp, including long inverted repeats at each extremity (10 kb).[89] The G/C content is 60%, one of the highest among the known giant viruses (Fig. 17.1). Gene prediction was performed in association to transcriptome sequencing, revealing 523 protein-coding genes and 3 tRNAs (Leu, Met, and Tyr). Spliceosomal introns were detected in 21 (4%) genes, mostly a single intron per gene.[89]

When mollivirus was discovered, 337 (64.4%) ORFans were detected in its genome.[89] Among the proteins with identifiable homologues, 93 (17.8%) are more similar to viruses, 50 (9.6%) to *A castellanii*, 22 (4.2%) to other eukaryotes, and 18 (3.4%) to prokaryotes.[89] Eighty-three predicted proteins have pandoravirus sequences as their best match, although they are only about 40% similar, including B-type DNA polymerase, a primase, helicases, and exonucleases. It is notable that the protein ml_347 (*Mollivirus sibericum*) is distantly similar (62.6% identical) to the MCP encoded by *A castellanii*. Other proteins predicted in the mollivirus genome are DNA-processing enzymes (polymerase, nuclease, helicase, etc.), deoxycytidylate deaminase, guanylate kinase, and ribonucleotide reductase. In conclusion, genomic analysis of the molliviruses indicates a new group of giant viruses related to pandoraviruses.[39,89]

Stages of Replication

Due to the dimensions of the virus particle, entry is likely to occur by phagocytosis in individual vacuoles, with the estimate of incorporation of up to 10 particles per *A castellanii* cell.[89,123] As described for other nucleocytoviruses, the internal membrane of the virion fuses with the membrane of the phagosome, and the interior of the particle is invaded by cytoplasm content (Fig. 17.10).[89,123] As mentioned, the ML_417 protein, present in the particle, has a fusion domain and might therefore be involved with uncoating.[89] There is migration of viral DNA to the nucleus of the amoeba, a phenomenon that is compatible with the absence of detection of transcriptional machinery in the virion.[89] Remarkably, the amoebas infected by mollivirus sibericum remain adhered and with typical morphology throughout the replication cycle. Analysis of *M sibericum* transcriptome revealed that, unlike what had been described for other dsDNA viruses, there is no temporally regulated gene expression over the cycle, because most genes are constantly expressed in early, intermediate, and late phases of the cycle.[89]

Although few mollivirus isolates have been described, there have been remarkable advances in characterizing the particle morphogenesis (Fig. 17.10).[39,89,123] At 8 hpi, the cell nucleus appears absent or disorganized, reduced in size, and loses its spherical aspect. A >3-µm viral factory is observed in the cytoplasm, occupying most of the cell. Most cellular organelles are displaced toward the cell periphery, and the mitochondria appear smaller and more elongated. Particles at different assembly phases are observed concomitantly.[89,123] Mollivirus particle assembly starts by a unique membrane precursor resembling a cisterna associated with a central flat pole (Fig. 17.10).[123] The assembly starts from this pole and extends toward symmetrical, open curling ends. The layers of capsid and tegument are gradually deposited starting from the flat pole. The development of the internal membrane appears to be associated with the recruitment of vesicles, which open upon contacting the ends of the membrane. As the internal membrane develops, viral core proteins are incorporated by the particle. At this stage, there is a cisternal element containing segregated ribosomes near the developing particle. The particle then develops, acquiring a spherical symmetry, but maintains an opening in an antipodal position to the flat pole.[123] Fibrillar material, possibly the genome, is then incorporated in the particle through this opening. Finally, the particle is closed. Virus particles are released in the supernatant of infected cells from 6 hpi onward. Although lysis of the infected cells was not described, it was hypothesized that the viral progeny is released by exocytosis—on average 250 particles per cell.[89,123]

Medusavirus

The first medusavirus described was discovered from a muddy freshwater sample collected from a hot spring in Japan.[185] This virus was isolated in *A castellanii*, and even though the virus causes cell lysis, part of the trophozoites turn into cystlike cells. This encystmentlike phenomenon has inspired authors to name the virus Acanthamoeba castellanii medusavirus, after Medusa, the character in ancient Greek mythology who turns onlookers to stone.[185] Recently, a new related virus has been described, named clandestinovirus.[133] This virus was isolated on *V vermiformis* from a wastewater sample collected in France, and despite differences in relation to the gene content, it was proposed that clandestinovirus is a member of the putative family "*Medusaviridae*."[133] Medusavirus and clandestinovirus present striking similarities, including an extensive set of histones in their virions, an intense gene exchange with amoebas, and the exploitation of the host nucleus, especially during the early and intermediate phases of infection.[133,185]

Virion Structure

Single-particle cryo-EM revealed that the medusavirus virion is a T = 277 icosahedron (h = 7; k = 12) and is ~260 nm in diameter (Fig. 17.11). The capsid is 8 nm thick and associated with surface spherical-headed spikes of ~14 nm in length. Each capsomers has a protruding spike. An inner membrane (6 nm thick) is observed in association with the capsid. The membrane wraps the 381-kb genome and core proteins of medusavirus (Fig. 17.1).[185]

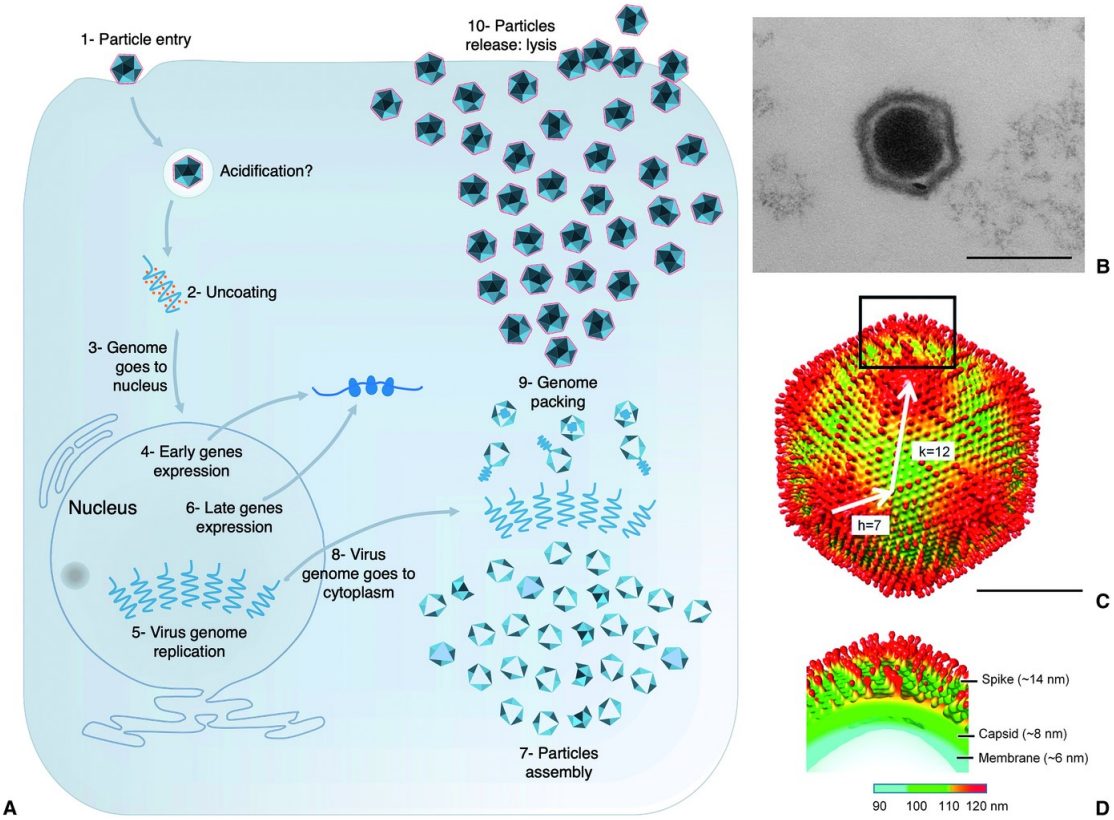


FIGURE 17.11 **Medusavirus replication cycle and structural features**. **A**. Schematic overview of the medusavirus replication cycle. 1-3—Medusavirus particles enter amoebas by an unknown mechanism. Virus genome and proteins are released into the host cytoplasm and go to the nucleus. 4-6—In the nucleus, virus early genes are transcribed by the cellular machinery, followed by genome replication and expression of late genes. 7-9—The assembly of virus particles occurs in the cytoplasm. Particles under different stages of assembly are usually observed in the cytoplasm. Concomitantly with the virus assembly, the mollivirus genome copies migrate from the nucleus to the cytoplasm and are then packed into the particles. 10—Newly formed medusavirus particles are released after cell lysis. **B**. Transmission electron microscopy of a medusavirus particle. Scale bar: 200 nm. (Courtesy of Dr. Masaharu Takemura, Tokyo University of Science, Japan.) **C**. Three-dimensional reconstruction of a medusavirus particle. The k and h values (used to calculate the T number) are indicated in the figure. The *black square* region is highlighted in panel **D**. Scale bar: 100 nm. **D**. Periphery of the medusavirus fivefold axis, highlighting the length of the spike, capsid, and membrane regions. For images in panels **C** and **D**, the *colors* indicate the distance from the center of the particle (scale at the bottom of panel **D**). (Images in panels **C** and **D** were adapted from Yoshikawa G, Blanc-Mathieu R, Song C, et al. Medusavirus, a novel large DNA virus discovered from hot spring water. *J Virol*. 2019;93(8):e02130-18. http://creativecommons.org/licenses/by/4.0/.)

The proteomic analysis of *A castellanii* medusavirus particles revealed 80 proteins, including >50 with unknown functions. Among the identified proteins, an MCP homolog is the most abundant.[185] In addition, DNA-packing ATPases, Rho transcription termination factors, and redox-active proteins were described in the particle. As a hallmark of medusaviruses, a set of viral-encoded core histones (H2A, H2B, H3, H4) are present in dissimilar abundances in the virion.[185] Transcription-related proteins were not found, explaining the medusavirus dependence on the host nucleus, especially during early phases of the cycle.

Clandestinovirus particles analyzed by transmission electron microscopy are 180-nm-wide icosahedrons. Future studies on particle proteomics and structure need to be performed to address its possible resemblance to medusavirus.[133]

Genome Structure and Organization

The *A castellanii* medusavirus genome is a linear 381 277 bp dsDNA molecule with a G/C content of 61.7% (Fig. 17.1).[185] 461 predicted protein-coding genes were identified, seven of which harbor spliceosomal introns. In addition, three tRNA-like sequences were found. The genome has a theoretical coding density of 89.5%. 279 (61%) predicted genes have no identified homologs (ORFans). Among the 182 (39%) genes with homologs, 115 presented eukaryotes as best matches, with 86 having their closest homologs in *A castellanii*. Interestingly, 57 genes were identified as candidates involved in horizontal gene transfer between medusavirus and *A castellanii*, including the MCP gene, which seems to have been transferred from the viral to amoeba genome.[185]

Identifiable motifs and functional domains were found in only 105 (23%) ORFs. Among the predicted proteins are enzymes related to DNA metabolism (including a family B DNA polymerase), transcription-related proteins, translation initial factor elF1, MCP, and A32-like DNA-packing ATPase. As mentioned, *A castellanii* medusavirus encodes homologs of all four core histones (H2A, H2B, H3, and H4) and the linker histone H1.[185] Medusavirus stheno, a strain described in 2021, has H3 and H4 fused into a single CDS.[184] Finally, the *A castellanii* medusavirus genome contains a putative cyclin B homolog, a putative metacaspase, and a homolog of the mitochondrial chaperone BCS1.[185]

The clandestinovirus genome is 581 987 bp with a G/C content of 43.5%[133] and encodes 617 predicted proteins and two tRNAs. 403 ORFs (65.3%) presented no identified homologs (ORFans). An extensive set of histones coding genes was also observed in clandestinovirus, including

H2B/H2A, H3, H4, and the linker H1/H5.[133]

Stages of Replication

As mentioned previously, medusavirus was described to replicate in *A castellanii*, while clandestinovirus was described to replicate in *V vermiformis*.[133,185] The entry pathways and genome uncoating mechanisms associated with medusavirus and clandestinovirus particles are unclear (Fig. 17.11). However, fluorescence *in situ* hybridization assays indicated that the genome of medusavirus migrates to the nucleus of the host in <1 hpi.[185] Medusavirus does not encode a DNA-dependent RNA polymerase or mRNA capping enzyme, which explains the need to access the host transcription machinery.[185] Temporal analysis of the medusavirus transcriptome revealed that 42 genes are expressed immediately early, including 35 genes with unknown functions, a linker H1 histone gene, and a poly-A polymerase regulatory subunit gene.[188]

From 2 hpi, the genome of medusavirus begins to be replicated in the host nucleus, in a region near the nucleolus (Fig. 17.11).[185] Early genes are expressed at this stage, including those related to nucleotide metabolism, DNA replication, recombination, and repair.[188] The amount of viral DNA in the nucleus increases considerably at 8 hpi.[185] After 8 hpi, intermediate and late viral genes are expressed, including the four core histones (H2A, H2B, H3, and H4), transcription factors, structural genes (such as MCP), and some translation factors.[188] Large amounts of virus particles are then assembled in the cytoplasm; at 8-10 hpi, they are empty or contain electron-lucent material. At this stage, the cellular nucleus remains morphologically intact but is filled with copies of virus genomes. From 14 hpi onward, copies of the virus genome migrate to the cytoplasm where they are packaged in particles.[185]

Similarly, clandestinovirus particles migrate to the nucleus of *V vermiformis* cells from 4 hpi, and by an unknown mechanism, the particles penetrate the nucleus.[133] The clandestinovirus induces the formation of a nuclear viral factory. Particles appear to be assembled and filled on the periphery of the viral factory, followed by particle migration to the cytoplasm.[133]

Medusavirus particles are released from 14 hpi onward, likely after host cell lysis.[185] Clandestinovirus particles are released by exocytosis from 16 hpi. Interestingly, infected cells become rounded, lose adhesion, but do not suffer lysis for at least 7 days.[133]

Perspectives

Research on giant viruses is expanding and experts from different disciplines unravel details about infection cycles, structures, host-virus relationships, evolution, metagenomics, and ecological aspects.[54] Nearly two decades after the serendipitous discovery of APMV, we know that giant viruses are ubiquitous and diverse pathogens of natural protist communities and that they interact with a variety of other microorganisms and mobile genetic elements. Yet, the majority of giant viruses still await discovery, requiring us to improve methods for their isolation and culture-independent characterization. Novel entities, such as yaravirus, mininucleoviruses, and meelsvirus, as well as thousands of novel viruses discovered by metagenomic give us a first taste of the richness and diversity of viruses within the kingdom *Bamfordvirae*.[20,28,146,151,159]

Acknowledgments

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REFERENCES

1. Abergel C, Legendre M, Claverie JM. The rapidly expanding universe of giant viruses: Mimivirus, Pandoravirus, Pithovirus and Mollivirus. FEMS Microbiol Rev. 2015;39(6):779-796.

2. Abrahão J, Silva L, Oliveira D, et al. Lack of evidence of mimivirus replication in human PBMCs. Microbes Infect. 2018;20(5):281-283.

3. Abrahão J, Silva L, Silva LS, et al. Tailed giant Tupanvirus possesses the most complete translational apparatus of the known virosphere. Nat Commun. 2018;9(1):749.

4. Abrahão JS, Araújo R, Colson P, et al. The analysis of translation-related gene set boosts debates around origin and evolution of mimiviruses. PLoS Genet. 2017;13(2):e1006532.

5. Agarkova I, Dunigan D, Gurnon J, et al. Chlorovirus-mediated membrane depolarization of Chlorella alters secondary active transport of solutes. J Virol. 2008;82(24):12181-12190.

6. Agarkova IV, Lane LC, Dunigan DD, et al. Identification of a chlorovirus PBCV-1 protein involved in degrading the host cell wall during virus infection. Viruses. 2021;13(5):782.

7. Aherfi S, Andreani J, Baptiste E, et al. A large open pangenome and a small core genome for giant pandoraviruses. Front Microbiol. 2018;9:1486.

8. Aherfi S, Brahim Belhaouari D, et al. Incomplete tricarboxylic acid cycle and proton gradient in Pandoravirus massiliensis: is it still a virus? ISME J. 2022;16(3):695-704.

9. Allen MJ, Howard JA, Lilley KS, et al. Proteomic analysis of the EhV-86 virion. Proc Natl Acad Sci U S A. 2008;6:11.

10. Allen MJ, Schroeder DC, Donkin A, et al. Genome comparison of two Coccolithoviruses. Virol J. 2006;3:15.

11. Andrade ACDSP, Rodrigues RAL, et al. Filling knowledge gaps for mimivirus entry, uncoating, and morphogenesis. J Virol. 2017;91(22):e01335-17.

12. Andreani J, Aherfi S, Bou Khalil JY, et al. Cedratvirus, a double-cork structured giant virus, is a distant relative of pithoviruses. Viruses. 2016;8(11):E300.

13. Andreani J, Khalil JYB, Baptiste E, et al. Orpheovirus IHUMI-LCC2: a new virus among the giant viruses. Front Microbiol. 2017;8:2643.

14. Andreani J, Khalil JYB, Sevvana M, et al. Pacmanvirus, a new giant icosahedral virus at the crossroads between Asfarviridae and faustoviruses. J Virol. 2017;91(14):e00212-17.

15. Andreani J, Schulz F, Di Pinto F, et al. Morphological and genomic features of the new Klosneuvirinae isolate fadolivirus IHUMI-VV54. Front Microbiol. 2021;12:719703.

16. Aoki K, Fukaya S, Takahashi H, et al. Marseilleviridae lineage B diversity and bunch formation inhibited by galactose. Microbes Environ. 2021;36(1):ME20139.

17. Arantes TS, Rodrigues RAL, Dos Santos Silva LK, et al. The large marseillevirus explores different entry pathways by forming giant infectious vesicles. J Virol. 2016;90(11):5246-5255.

18. Arslan D, Legendre M, Seltzer V, et al. Distant Mimivirus relative with a larger genome highlights the fundamental features of Megaviridae. Proc Natl Acad Sci. 2011;108(42):17486-17491.

19. Aylward FO, Moniruzzaman M, Ha AD, et al. A phylogenomic framework for charting the diversity and evolution of giant viruses. PLoS Biol. 2021;19(10):e3001430.

20. Bäckström D, Yutin N, Jørgensen SL, et al. Virus genomes from deep sea sediments expand the ocean megavirome and support independent origins of viral gigantism. MBio. 2019;10(2):e02497-18.

21. Bajrai LH, Benamar S, Azhar EI, et al. Kaumoebavirus, a new virus that clusters with Faustoviruses and Asfarviridae. Viruses. 2016;8(11):E278.

22. Bajrai LH, Mougari S, Andreani J, et al. Isolation of yasminevirus, the first member of klosneuvirinae isolated in coculture with Vermamoeba vermiformis, demonstrates an extended arsenal of translational apparatus components. J Virol. 2019;94(1):e01534-19.

23. Bamford DH, Burnett RM, Stuart DI. Evolution of viral structure. Theor Popul Biol. 2002;61(4):461-470.

24. Baudoux AC, Brussaard CPD. Characterization of different viruses infecting the marine harmful algal bloom species Phaeocystis globosa. Virology. 2005;341(1):80-90.

25. Benamar S, Reteno DGI, Bandaly V, et al. Faustoviruses: comparative genomics of new megavirales family members. Front Microbiol. 2016;7:3.

26. Blanca L, Christo-Foroux E, Rigou S, et al. Comparative analysis of the circular and highly asymmetrical Marseilleviridae genomes. Viruses. 2020;12(11):1270.

27. Blanc-Mathieu R, Dahle H, Hofgaard A, et al. A persistent giant algal virus, with a unique morphology, encodes an unprecedented number of genes involved in energy metabolism. J Virol. 2021;95(8):e02446-20.

28. Boratto P, Albarnaz JD, Almeida GM, et al. *Acanthamoeba polyphaga* mimivirus prevents amoebal encystment-mediating serine proteinase expression and circumvents cell encystment. J Virol. 2015;89(5):2962-2965.

29. Boratto PVM, Arantes TS, Silva LCF, et al. Niemeyer virus: a new mimivirus group a isolate harboring a set of duplicated aminoacyl-tRNA synthetase genes. Front Microbiol. 2015;6:1256.

30. Boratto PVM, Dornas FP, da Silva LCF, et al. Analyses of the kroon virus major capsid gene and its transcript highlight a distinct pattern of gene evolution and splicing among mimiviruses. J Virol. 2018;92(2):e01782-17.

31. Borges I, Rodrigues RAL, Dornas FP, et al. Trapping the enemy: Vermamoeba vermiformis circumvents Faustovirus mariensis dissemination by enclosing viral progeny inside cysts. J Virol. 2019;93(14):e00312-19.

32. Boyer M, Azza S, Barrassi L, et al. Mimivirus shows dramatic genome reduction after intraamoebal culture. Proc Natl Acad Sci U S A. 2011;108(25):10296-10301.

33. Boyer M, Madoui MA, Gimenez G, et al. Phylogenetic and phyletic studies of informational genes in genomes highlight existence of a 4 domain of life including giant viruses. PLoS One. 2010;5(12):e15530.

34. Boyer M, Yutin N, Pagnier I, et al. Giant Marseillevirus highlights the role of amoebae as a melting pot in emergence of chimeric microorganisms. Proc Natl Acad Sci U S A. 2009;106(51):21848-21853.

35. Brahim Belhaouari D, Baudoin JP, Gnankou F, et al. Evidence of a cellulosic layer in pandoravirus massiliensis tegument and the mystery of the genetic support of its biosynthesis. Front Microbiol. 2019;10:2932.

36. Brussaard CPD, Short SM, Frederickson CM, et al. Isolation and phylogenetic analysis of novel viruses infecting the phytoplankton Phaeocystis globosa (Prymnesiophyceae). Appl Environ Microbiol. 2004;70(6):3700-3705.

37. Cherif Louazani A, Baptiste E, Levasseur A, et al. Faustovirus E12 transcriptome analysis reveals complex splicing in capsid gene. Front Microbiol. 2018;9:2534.

38. Cherrier MV, Kostyuchenko VA, Xiao C, et al. An icosahedral algal virus has a complex unique vertex decorated by a spike. Proc Natl Acad Sci U S A. 2009;106(27):11085-11089.

39. Christo-Foroux E, Alempic JM, Lartigue A, et al. Characterization of mollivirus kamchatka, the first modern representative of the proposed Molliviridae family of giant viruses. J Virol. 2020;94(8):e01997-19.

40. Claverie JM, Abergel C, Ogata H. Mimivirus. Curr Top Microbiol Immunol. 2009;328:89-121.

41. Colson P, La Scola B, Levasseur A, et al. Mimivirus: leading the way in the discovery of giant viruses of amoebae. Nat Rev Microbiol. 2017;15(4):243-254.

42. Deeg CM, Chow CET, Suttle CA. The kinetoplastid-infecting Bodo saltans virus (BsV), a window into the most abundant giant viruses in the sea. Elife. 2018;7:e33014.

43. Delaroque N, Müller DG, Bothe G, et al. The complete DNA sequence of the ectocarpus siliculosus virus EsV-1 genome. Virology. 2001;287(1):112-132.

44. Derelle E, Monier A, Cooke R, et al. Diversity of viruses infecting the green microalga Ostreococcus lucimarinus. J Virol. 2015;89(11):5812-5821.

45. Desnues C, La Scola B, Yutin N, et al. Provirophages and transpovirons as the diverse mobilome of giant viruses. Proc Natl Acad Sci U S A. 2012;109(44):18078-18083.

46. Dodds JA, Cole A. Microscopy and biology of Uronema gigas, a filamentous eucaryotic green alga, and its associated tailed virus-like particle. Virology. 1980;100(1):156-165.

47. Dornas FP, Assis FL, Aherfi S, et al. A Brazilian marseillevirus is the founding member of a lineage in family Marseilleviridae. Viruses. 2016;8(3):76.

48. Dos Santos RN, Campos FS, Medeiros de Albuquerque NR, et al. A new marseillevirus isolated in Southern Brazil from Limnoperna fortunei. Sci Rep. 2016;6:35237.

49. Duponchel S, Fischer MG. Viva lavidaviruses! Five features of virophages that parasitize giant DNA viruses. PLoS Pathog. 2019;15(3):e1007592.

50. Fabre E, Jeudy S, Santini S, et al. Noumeavirus replication relies on a transient remote control of the host nucleus. Nat Commun. 2017;8:15087.

51. Fang Q, Zhu D, Agarkova I, et al. Near-atomic structure of a giant virus. Nat Commun. 2019;10(1):388.

52. Filée J. Route of NCLDV evolution: the genomic accordion. Curr Opin Virol. 2013;3(5):595-599.

53. Finke J, Winget D, Chan A, et al. Variation in the genetic repertoire of viruses infecting micromonas pusilla reflects horizontal gene transfer and links to their environmental distribution. Viruses. 2017;9(5):116.

54. Fischer MG. Giant viruses come of age. Curr Opin Microbiol. 2016;31:50-57.

55. Fischer MG, Allen MJ, Wilson WH, et al. Giant virus with a remarkable complement of genes infects marine zooplankton. Proc Natl Acad Sci U S A. 2010;107(45):19508-19513.

56. Fischer MG, Hackl T. Host genome integration and giant virus-induced reactivation of the virophage mavirus. Nature. 2016;540(7632):288-291.

57. Fischer MG, Kelly I, Foster LJ, et al. The virion of *Cafeteria roenbergensis* virus (CroV) contains a complex suite of proteins for transcription and DNA repair. Virology. 2014;466-467:82-94.

58. Fischer MG, Suttle CA. A virophage at the origin of large DNA transposons. Science. 2011;332(6026):231-234.

59. Fitzgerald LA, Boucher PT, Yanai-Balser GM, et al. Putative gene promoter sequences in the chlorella viruses. Virology. 2008;380(2):388-393.

60. Fraenkel-Conrat H. Newly Characterized Protist and Invertebrate Viruses. Springer Science & Business Media; 2012.

61. Fridmann-Sirkis Y, Milrot E, Mutsafi Y, et al. Efficiency in complexity: composition and dynamic nature of mimivirus replication factories. J Virol. 2016;90(21):10039-10047.

62. Fukaya S, Takemura M. Kinetic analysis of *Acanthamoeba castellanii* infected with giant viruses quantitatively revealed process of morphological and behavioral changes in host cells. Microbiol Spectr. 2021;9(1):e00368-21.

63. Gaia M, Benamar S, Boughalmi M, et al. Zamilon, a novel virophage with Mimiviridae host specificity. PLoS One. 2014;9(4):e94923.

64. Gallot-Lavallée L, Blanc G, Claverie JM. Comparative genomics of chrysochromulina ericina virus and other microalga-infecting large DNA viruses highlights their intricate evolutionary relationship with the established Mimiviridae family. J Virol. 2017;91(14):e00230-17.

65. Gann ER, Xian Y, Abraham PE, et al. Structural and proteomic studies of the Aureococcus anophagefferens virus demonstrate a global distribution of virus-encoded carbohydrate processing. Front Microbiol. 2020;11:2047.

66. Geballa-Koukoulas K, Andreani J, La Scola B, et al. The Kaumoebavirus LCC10 genome reveals a unique gene strand bias among “Extended Asfarviridae.” Viruses. 2021;13(2):148.

67. Geballa-Koukoulas K, Boudjemaa H, Andreani J, et al. Comparative genomics unveils regionalized evolution of the faustovirus genomes.

Viruses. 2020;12(5):577.

68. Gimenes MV, Zanotto PM, Suttle CA, et al. Phylodynamics and movement of Phycodnaviruses among aquatic environments. ISME J. 2012;6(2):237-247.

69. Ha AD, Moniruzzaman M, Aylward FO. High transcriptional activity and diverse functional repertoires of hundreds of giant viruses in a coastal marine system. mSystems. 2021;6(4):e0029321.

70. Hingamp P, Grimsley N, Acinas SG, et al. Exploring nucleo-cytoplasmic large DNA viruses in Tara Oceans microbial metagenomes. ISME J. 2013;7(9):1678-1695.

71. Hosokawa N, Takahashi H, Aoki K, et al. Draft genome sequence of pandoravirus japonicus isolated from the Sabaishi River, Niigata, Japan. Microbiol Resour Announc. 2021;10(19):e00365-21.

72. Iyer LM, Aravind L, Koonin EV. Common origin of four diverse families of large eukaryotic DNA viruses. J Virol. 2001;75(23):11720-11734.

73. Jeudy S, Bertaux L, Alempic JM, et al. Exploration of the propagation of transpovirons within Mimiviridae reveals a unique example of commensalism in the viral world. ISME J. 2020;14(3):727-739.

74. Johannessen TV, Bratbak G, Larsen A, et al. Characterisation of three novel giant viruses reveals huge diversity among viruses infecting Prymnesiales (Haptophyta). Virology. 2015;476:180-188.

75. Kijima S, Delmont TO, Miyazaki U, et al. Discovery of viral myosin genes with complex evolutionary history within plankton. Front Microbiol. 2021;12:1450.

76. Klose T, Reteno DG, Benamar S, et al. Structure of faustovirus, a large dsDNA virus. Proc Natl Acad Sci U S A. 2016;113(22):6206-6211.

77. Koonin EV, Dolja VV, Krupovic M, et al. Global organization and proposed megataxonomy of the virus world. Microbiol Mol Biol Rev. 2020;84(2):e00061-19.

78. Krupovic M, Koonin EV. Polintons: a hotbed of eukaryotic virus, transposon and plasmid evolution. Nat Rev Microbiol. 2015;13(2):105-115.

79. Krupovic M, Yutin N, Koonin E. Evolution of a major virion protein of the giant pandoraviruses from an inactivated bacterial glycoside hydrolase. Virus Evol. 2020;6(2):veaa059.

80. Kuznetsov YG, Klose T, Rossmann M, et al. Morphogenesis of mimivirus and its viral factories: an atomic force microscopy study of infected cells. J Virol. 2013;87(20):11200-11213.

81. Kuznetsov YG, Xiao C, Sun S, et al. Atomic force microscopy investigation of the giant mimivirus. Virology. 2010;404(1):127-137.

82. La Scola B, Audic S, Robert C, et al. A giant virus in amoebae. Science. 2003;299(5615):2033.

83. La Scola B, Desnues C, Pagnier I, et al. The virophage as a unique parasite of the giant mimivirus. Nature. 2008;455(7209):100-104.

84. La Scola B, Marrie TJ, Auffray JP, et al. Mimivirus in pneumonia patients. Emerg Infect Dis. 2005;11(3):449-452.

85. Legendre M, Alempic JM, Philippe N, et al. Pandoravirus celtis illustrates the microevolution processes at work in the giant Pandoraviridae genomes. Front Microbiol. 2019;10:430.

86. Legendre M, Audic S, Poirot O, et al. mRNA deep sequencing reveals 75 new genes and a complex transcriptional landscape in Mimivirus. Genome Res. 2010;20(5):664-674.

87. Legendre M, Bartoli J, Shmakova L, et al. Thirty-thousand-year-old distant relative of giant icosahedral DNA viruses with a pandoravirus morphology. Proc Natl Acad Sci U S A. 2014;111(11):4274-4279.

88. Legendre M, Fabre E, Poirot O, et al. Diversity and evolution of the emerging Pandoraviridae family. Nat Commun. 2018;9(1):2285.

89. Legendre M, Lartigue A, Bertaux L, et al. In-depth study of Mollivirus sibericum, a new 30,000-y-old giant virus infecting Acanthamoeba. Proc Natl Acad Sci U S A. 2015;112(38):E5327-E5335.

90. Levasseur A, Bekliz M, Chabrière E, et al. MIMIVIRE is a defence system in mimivirus that confers resistance to virophage. Nature. 2016;531(7593):249-252.

91. Lundholm IV, Sellberg JA, Ekeberg T, et al. Considerations for three-dimensional image reconstruction from experimental data in coherent diffractive imaging. IUCrJ. 2018;5(5):531-541.

92. Mackinder LCM, Worthy CA, Biggi G, et al. A unicellular algal virus, *Emiliania huxleyi* virus 86, exploits an animal-like infection strategy. J Gen Virol. 2009;90(Pt 9):2306-2316.

93. Maier I, Müller DG. Virus binding to brown algal spores and gametes visualized by DAPI fluorescence microscopy. Phycologia. 1998;37(1):60-63.

94. Maier I, Müller DG, Katsaros C. Entry of the DNA virus, Ectocarpus fasciculatus virus type 1 (Phycodnaviridae), into host cell cytosol and nucleus. Phycol Res. 2002;50(3):227-231.

95. Maier I, Wolf S, Delaroque N, et al. A DNA virus infecting the marine brown alga *Pilayella littoralis* (Ectocarpales, Phaeophyceae) in culture. Eur J Phycol. 1998;33(3):213-220.

96. Meints RH, Lee K, Van Etten JL. Assembly site of the virus PBCV-1 in a Chlorella-like green alga: ultrastructural studies. Virology. 1986;154(1):240-245.

97. Meints RH, Van Etten JL, Kuczmarski D, et al. Viral infection of the symbiotic chlorella-like alga present in Hydra viridis. Virology. 1981;113(2):698-703.

98. Milrot E, Mutsafi Y, Fridmann-Sirkis Y, et al. Virus-host interactions: insights from the replication cycle of the large Paramecium bursaria chlorella virus. Cell Microbiol. 2016;18(1):3-16.

99. Milrot E, Shimoni E, Dadosh T, et al. Structural studies demonstrating a bacteriophage-like replication cycle of the eukaryote-infecting Paramecium bursaria chlorella virus-1. PLoS Pathog. 2017;13(8):e1006562.

100. Monier A, Pagarete A, de Vargas C, et al. Horizontal gene transfer of an entire metabolic pathway between a eukaryotic alga and its DNA virus. Genome Res. 2009;19(8):1441-1449.

101. Mordecai GJ, Verret F, Highfield A, et al. Schrödinger's Cheshire cat: are haploid *Emiliania huxleyi* cells resistant to viral infection or not? Viruses. 2017;9(3):51.

102. Mougari S, Bekliz M, Abrahao J, et al. Guarani virophage, a new sputnik-like isolate from a Brazilian lake. Front Microbiol. 2019;10:1003.

103. Mougari S, Chelkha N, Sahmi-Bounsiar D, et al. A virophage cross-species infection through mutant selection represses giant virus propagation, promoting host cell survival. Commun Biol. 2020;3(1):248.

104. Mutsafi Y, Shimoni E, Shimon A, et al. Membrane assembly during the infection cycle of the giant Mimivirus. PLoS Pathog. 2013;9(5):e1003367.

105. Mutsafi Y, Zauberman N, Sabanay I, Minsky A. Vaccinia-like cytoplasmic replication of the giant Mimivirus. Proc Natl Acad Sci U S A. 2010;107(13):5978-5982.

106. Needham DM, Yoshizawa S, Hosaka T, et al. A distinct lineage of giant viruses brings a rhodopsin photosystem to unicellular marine predators. Proc Natl Acad Sci. 2019;116(41):20574-20583.

107. Nissimov JI, Napier JA, Kimmance SA, et al. Permanent draft genomes of four new coccolithoviruses: EhV-18, EhV-145, EhV-156 and EhV-164. Mar Genomics. 2014;15:7-8.

108. Nissimov JI, Pagarete A, Ma F, et al. Coccolithoviruses: a review of cross-kingdom genomic thievery and metabolic thuggery. Viruses. 2017;9(3):52.

109. Ogata H, Raoult D, Claverie JM. A new example of viral intein in Mimivirus. Virol J. 2005;2(1):8.

110. Ogura Y, Hayashi T, Ueki S. Complete genome sequence of a phycodnavirus, heterosigma akashiwo virus strain 53. Genome Announc. 2016;4(6):e01279-16.

111. Okamoto K, Miyazaki N, Reddy HKN, et al. Cryo-EM structure of a Marseilleviridae virus particle reveals a large internal microassembly. Virology. 2018;516:239-245.

112. Okamoto K, Miyazaki N, Song C, et al. Structural variability and complexity of the giant Pithovirus sibericum particle revealed by high-voltage electron cryo-tomography and energy-filtered electron cryo-microscopy. Sci Rep. 2017;7(1):13291.

113. Oliveira G, Silva L, Leão T, et al. Tupanvirus-infected amoebas are induced to aggregate with uninfected cells promoting viral dissemination. Sci Rep. 2019;9(1):183.

114. Oliveira GP, de Aquino ILM, Luiz APMF, et al. Putative promoter motif analyses reinforce the evolutionary relationships among Faustoviruses, Kaumoebavirus, and Asfarvirus. Front Microbiol. 2018;9:1041.

115. Oliveira GP, Lima MT, Arantes TS, et al. The investigation of promoter sequences of marseilleviruses highlights a remarkable abundance of the AAATATTT motif in intergenic regions. J Virol. 2017;91(21):e01088-17.

116. Pagarete A, Allen MJ, Wilson WH, et al. Host-virus shift of the sphingolipid pathway along an *Emiliania huxleyi* bloom: survival of the fattest. Environ Microbiol. 2009;11(11): 2840-2848.

117. Dunigan DD, Cerny RL, Bauman AT, et al. Paramecium bursaria chlorella virus 1 proteome reveals novel architectural and regulatory features of a giant virus. J Virol. 2012;86:8821-8834.

118. Pereira Andrade ACDS, Victor de Miranda Boratto P, Rodrigues RAL, et al. New isolates of Pandoraviruses: contribution to the study of replication cycle steps. J Virol. 2019;93(5):e01942-18.

119. Philippe N, Legendre M, Doutre G, et al. Pandoraviruses: amoeba viruses with genomes up to 2.5 Mb reaching that of parasitic eukaryotes. Science. 2013;341(6143):281-286.

120. Piacente F, De Castro C, Jeudy S, et al. The rare sugar N-acetylated viosamine is a major component of Mimivirus fibers. J Biol Chem. 2017;292(18):7385-7394.

121. Pickett-Heaps JD. A possible virus infection in the Green Alga Oedogonium 1. J Phycol. 1972;8(1):44-47.

122. Pienaar RN. Virus-like particles in three species of phytoplankton from San Juan Island, Washington. Phycologia. 1976;15(2):185-190.

123. Quemin ER, Corroyer-Dulmont S, Baskaran A, et al. Complex membrane remodeling during virion assembly of the 30,000-year-old

Mollivirus Sibericum. J Virol. 2019;93(13):e00388-19.

124. Raoult D, Audic S, Robert C, et al. The 1.2-megabase genome sequence of Mimivirus. Science. 2004;306(5700):1344-1350.

125. Renesto P, Abergel C, Decloquement P, et al. Mimivirus giant particles incorporate a large fraction of anonymous and unique gene products. J Virol. 2006;80(23):11678-11685.

126. Reteno DG, Benamar S, Khalil JB, et al. Faustovirus, an asfarvirus-related new lineage of giant viruses infecting amoebae. J Virol. 2015;89(13):6585-6594.

127. Rodrigues RA, de Souza FG, de Azevedo BL, et al. The morphogenesis of different giant viruses as additional evidence for a common origin of Nucleocytoviricota. Curr Opin Virol. 2021;49:102-110.

128. Rodrigues RAL, Andreani J, Andrade ACDSP, et al. Morphologic and genomic analyses of new isolates reveal a second lineage of cedratviruses. J Virol. 2018;92(13):e00372-18.

129. Rodrigues RAL, Arantes TS, Oliveira GP, et al. Chapter four—the complex nature of Tupanviruses. In: Kielian M, Mettenleiter TC, Roossinck MJ, eds. Adv Virus Res. Vol 103. Academic Press; 2019:135-166.

130. Rodrigues RAL, Louazani AC, Picorelli A, et al. Analysis of a marseillevirus transcriptome reveals temporal gene expression profile and host transcriptional shift. Front Microbiol. 2020;11:651.

131. Rodrigues RAL, Queiroz VF, Ghosh J, et al. Functional genomic analyses reveal an open pan-genome for the chloroviruses and a potential for genetic innovation in new isolates. J Virol. 2022;96(2):e0136721.

132. Rodrigues RAL, dos Santos Silva LK, Dornas FP, et al. Mimivirus fibrils are important for viral attachment to the microbial world by a diverse glycoside interaction repertoire. J Virol. 2015;89(23):11812-11819.

133. Rolland C, Andreani J, Sahmi-Bounsiar D, et al. Clandestinovirus: a giant virus with chromatin proteins and a potential to manipulate the cell cycle of its host Vermamoeba vermiformis. Front Microbiol. 2021;12:715608.

134. Rose SL, Fulton JM, Brown CM, et al. Isolation and characterization of lipid rafts in *Emiliania huxleyi*: a role for membrane microdomains in host-virus interactions. Environ Microbiol. 2014;16(4):1150-1166.

135. Rosenwasser S, Sheyn U, Frada MJ, et al. Unmasking cellular response of a bloom-forming alga to viral infection by resolving expression profiles at a single-cell level. PLoS Pathog. 2019;15(4):e1007708.

136. Roux S, Chan LK, Egan R, et al. Ecogenomics of virophages and their giant virus hosts assessed through time series metagenomics. Nat Commun. 2017;8(1):858.

137. Rowe JM, Dunlap JR, Gobler CJ, et al. Isolation of a non-phage-like lytic virus infecting Aureococcus anophagefferens. J Phycol. 2008;44(1):71-76.

138. Sahmi-Bounsiar D, Rolland C, Aherfi S, et al. Marseilleviruses: an update in 2021. Front Microbiol. 2021;12:648731.

139. Sakhaee F, Vaziri F, Bahramali G, et al. Pulmonary infection related to mimivirus in patient with primary ciliary dyskinesia. Emerg Infect Dis. 2020;26(10):2524-2526.

140. Sandaa RA, Heldal M, Castberg T, et al. Isolation and characterization of two viruses with large genome size infecting Chrysochromulina ericina (Prymnesiophyceae) and Pyramimonas orientalis (Prasinophyceae). Virology. 2001;290(2):272-280.

141. Schatz D, Rosenwasser S, Malitsky S, et al. Communication via extracellular vesicles enhances viral infection of a cosmopolitan alga. Nat Microbiol. 2017;2(11):1485-1492.

142. Scheid P, Zöller L, Pressmar S, et al. An extraordinary endocytobiont in Acanthamoeba sp. isolated from a patient with keratitis. Parasitol Res. 2008;102(5):945-950.

143. Schrad JR, Abrahão JS, Cortines JR, et al. Structural and proteomic characterization of the initiation of giant virus infection. Cell. 2020;181(5):1046-1061.e6.

144. Schroeder DC, Oke J, Malin G, et al. Coccolithovirus (Phycodnaviridae): characterisation of a new large dsDNA algal virus that infects *Emiliania huxleyi*. Arch Virol. 2002;147(9):1685-1698.

145. Schroeder DC, Park Y, Yoon HM, et al. Genomic analysis of the smallest giant virus—Feldmannia sp. virus 158. Virology. 2009;384(1):223-232.

146. Schulz F, Roux S, Paez-Espino D, et al. Giant virus diversity and host interactions through global metagenomics. Nature. 2020;578(7795):432-436.

147. Schulz F, Yutin N, Ivanova NN, et al. Giant viruses with an expanded complement of translation system components. Science. 2017;356(6333):82-85.

148. Schvarcz CR, Steward GF. A giant virus infecting green algae encodes key fermentation genes. Virology. 2018;518:423-433.

149. Seibert MM, Ekeberg T, Maia FRNC, et al. Single mimivirus particles intercepted and imaged with an X-ray laser. Nature. 2011;470(7332):78-81.

150. Seitzer P, Jeanniard A, Ma F, et al. Gene gangs of the chloroviruses: conserved clusters of collinear monocistronic genes. Viruses.

2018;10(10):576.

151. Shinn GL, Bullard BL. Ultrastructure of Meelsvirus: a nuclear virus of arrow worms (phylum Chaetognatha) producing giant “tailed” virions. PLoS One. 2018;13(9):e0203282.

152. Silva LCF, Rodrigues RAL, Oliveira GP, et al. Microscopic analysis of the tupanvirus cycle in Vermamoeba vermiformis. Front Microbiol. 2019;10:671.

153. Silva LKDS, Andrade ACDSP, Dornas FP, et al. Cedratvirus getuliensis replication cycle: an in-depth morphological analysis. Sci Rep. 2018;8(1):4000.

154. Sobhy H, Scola BL, Pagnier I, et al. Identification of giant Mimivirus protein functions using RNA interference. Front Microbiol. 2015;6:345.

155. Souza F, Rodrigues R, Reis E, et al. In-depth analysis of the replication cycle of Orpheovirus. Virol J. 2019;16(1):158.

156. Stepanova OA, Boĭko AL, Shcherbatenko IS. Computational genome analysis of three marine algoviruses. Mikrobiol Z. 2013;75(5):76-81.

157. Strasser P, Zhang YP, Rohozinski J, et al. The termini of the chlorella virus PBCV-1 genome are identical 2.2-kbp inverted repeats. Virology. 1991;180(2):763-769.

158. Suárez C, Welsch S, Chlanda P, et al. Open membranes are the precursors for assembly of large DNA viruses. Cell Microbiol. 2013;15(11):1883-1895.

159. Subramaniam K, Behringer DC, Bojko J, et al. A new family of DNA viruses causing disease in crustaceans from diverse aquatic Biomes. MBio. 2020;11(1):e02938-19.

160. Suhre K, Audic S, Claverie JM. Mimivirus gene promoters exhibit an unprecedented conservation among all eukaryotes. Proc Natl Acad Sci U S A. 2005;102(41):14689-14693.

161. Sun S, La Scola B, Bowman VD, et al. Structural studies of the Sputnik virophage. J Virol. 2010;84(2):894-897.

162. Suzan-Monti M, Scola BL, Barrassi L, et al. Ultrastructural characterization of the giant volcano-like virus factory of *Acanthamoeba polyphaga* Mimivirus. PLoS One. 2007;2(3):e328.

163. Takahashi H, Fukaya S, Song C, et al. Morphological and taxonomic properties of the newly isolated Cotonvirus japonicus, a new lineage of the subfamily Megavirinae. J Virol. 2021;95(18):e00919-21.

164. Thomas V, Bertelli C, Collyn F, et al. Lausannevirus, a giant amoebal virus encoding histone doublets. Environ Microbiol. 2011;13(6):1454-1466.

165. Trainic M, Koren I, Sharoni S, et al. Infection dynamics of a bloom-forming alga and its virus determine airborne coccolith emission from seawater. iScience. 2018;6:327-335.

166. Valencia-Sánchez MI, Abini-Agbomson S, et al. The structure of a virus-encoded nucleosome. Nat Struct Mol Biol. 2021;28(5):413-417.

167. Van Etten JL, Agarkova IV, Dunigan DD. Chloroviruses. Viruses. 2019;12(1):20.

168. Van Etten JL, Burbank DE, Kuczmarski D, et al. Virus infection of culturable chlorella-like algae and development of a plaque assay. Science. 1983;219(4587):994-996.

169. Van Etten JL, Burbank DE, Xia Y, et al. Growth cycle of a virus, PBCV-1, that infects Chlorella-like algae. Virology. 1983;126(1):117-125.

170. Van Etten JL, Dunigan DD, Nagasaki K, et al. Phycodnaviruses (Phycodnaviridae). In: Encyclopedia of Virology. Elsevier; 2021:687-695.

171. Van Etten JL, Gurnon JR, Yanai-Balser GM, et al. Chlorella viruses encode most, if not all, of the machinery to glycosylate their glycoproteins independent of the endoplasmic reticulum and Golgi. Biochim Biophys Acta. 2010;1800(2):152-159.

172. Van Etten JL, Meints RH. Giant viruses infecting algae. Annu Rev Microbiol. 1999;53: 447-494.

173. Van Etten JL, Meints RH, Burbank DE, et al. Isolation and characterization of a virus from the intracellular green alga symbiotic with Hydra viridis. Virology. 1981;113(2): 704-711.

174. Van Etten JL, Meints RH, Kuczmarski D, et al. Viruses of symbiotic Chlorella-like algae isolated from Paramecium bursaria and Hydra viridis. Proc Natl Acad Sci U S A. 1982;79(12):3867-3871.

175. Weisman RA, Korn ED. Phagocytosis of latex beads by Acanthamoeba. I. Biochemical properties. Biochemistry. 1967;6(2):485-497.

176. Weynberg KD, Allen MJ, Gilg IC, et al. Genome sequence of ostreococcus tauri virus OtV-2 throws light on the role of picoeukaryote niche separation in the ocean. J Virol. 2011;85(9):4520-4529.

177. Wilson WH, Schroeder DC, Allen MJ, et al. Complete genome sequence and lytic phase transcription profile of a Coccolithovirus. Science. 2005;309(5737):1090-1092.

178. Xian Y, Avila R, Pant A, et al. The role of tape measure protein in nucleocytoplasmic large DNA virus capsid assembly. Viral Immunol. 2021;34(1):41-48.

179. Xiao C, Fischer MG, Bolotaulo DM, et al. Cryo-EM reconstruction of the Cafeteria roenbergensis virus capsid suggests novel assembly

pathway for giant viruses. Sci Rep. 2017;7(1):5484.

180. Xiao C, Kuznetsov YG, Sun S, et al. Structural studies of the giant mimivirus. PLoS Biol. 2009;7(4):e1000092.

181. Yamada T, Chuchird N, Kawasaki T, et al. Chlorella viruses as a source of novel enzymes. J Biosci Bioeng. 1999;88(4):353-361.

182. Yan X, Chipman PR, Castberg T, et al. The marine algal virus PpV01 has an icosahedral capsid with T=219 quasisymmetry. J Virol. 2005;79(14):9236-9243.

183. Yoosuf N, Yutin N, Colson P, et al. Related giant viruses in distant locations and different habitats: *Acanthamoeba polyphaga* moumouvirus represents a third lineage of the Mimiviridae that is close to the megavirus lineage. Genome Biol Evol. 2012;4(12): 1324-1330.

184. Yoshida K, Zhang R, Garcia KG, et al. Draft genome sequence of medusavirus stheno, isolated from the Tatakai river of Uji, Japan. Microbiol Resour Announc. 2021;10(1):e01323-20.

185. Yoshikawa G, Blanc-Mathieu R, Song C, et al. Medusavirus, a novel large DNA virus discovered from hot spring water. J Virol. 2019;93(8):e02130-18.

186. Yuan Y, Gao M. Jumbo bacteriophages: an overview. Front Microbiol. 2017;8:403.

187. Zauberman N, Mutsafi Y, Halevy DB, et al. Distinct DNA exit and packaging portals in the virus *Acanthamoeba polyphaga* mimivirus. PLoS Biol. 2008;6(5):e114.

188. Zhang R, Endo H, Takemura M, et al. RNA sequencing of Medusavirus suggests remodeling of the host nuclear environment at an early infection stage. Microbiol Spectr. 2021;9(2):e0006421.

189. Zhang X, Sun S, Xiang Y, et al. Structure of Sputnik, a virophage, at 3.5-Å resolution. Proc Natl Acad Sci U S A. 2012;109(45):18431-18436.

190. Zhang XA, Zhu T, Zhang PH, et al. Lack of mimivirus detection in patients with respiratory disease, China. Emerg Infect Dis. 2016;22(11):2011-2012.

191. Zhang Y, Strasser P, Grabherr R, et al. Hairpin loop structure at the termini of the chlorella virus PBCV-1 genome. Virology. 1994;202(2):1079-1082.

CHAPTER 18

Plant Viruses

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Current plant virus epidemics

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Plant viruses and biotechnology

Epilogue

Acknowledgments

Plant viruses are exceptional model systems with which to address universal concepts in virus replication, translation, intracellular movement, and other common aspects of virus infection cycles. Plant virus investigators uncovered the vascular super highway as a conduit for long distance intercellular movement of RNAs and unveiled the existence of RNA silencing, which controls gene expression, development, intercellular communication, and pathogen targeting. Plant viruses are also critical concerns for global food security, responsible for over 30 billion US dollars in annual losses.[119] Current viral epidemics threaten the world's rice production and supply of cacao, coffee, and other commodities of critical importance to the countries that cultivate them (and the consumers that require them), making the study of plant viruses and the development of therapeutics increasingly important as there are currently no treatments available for any virus-infected plant.

Recent reports have revealed that plant viruses are much more than just pathogens. Viruses can exist in mutualistic relationships with their insect vectors and host plants and virus capsids are emerging as important components of nanotechnology with boundless applications for human and animal health. Virus VIGS (virus-induced gene silencing) vectors are effective tools for genetic research by quickly and efficiently suppressing gene expression and may also be the long-sought silver bullets for controlling pathogens that are decimating entire industries by serving as delivery vehicles for siRNAs in both annual crops and long-lived trees and vines. This chapter cannot hope to cover even a small fraction of the exciting research being conducted on plant viruses in labs throughout the world, and we regret the many outstanding omissions. Rather, we have tried to give an overview of the current state of the field and point to some of the recent developments in plant-virus-vector interactions that are revolutionizing our understanding of these important pathogens. We refer readers to the excellent chapter from W. Allen Miller and Steven Whitham on Plant Viruses in the previous edition of Field's Virology for a history of the field and basic information on plant virus genome organization and material introductory to some of the information presented here.

Ecology of Plant Viruses

Viruses and plants are major occupants of our ecosystem. Identified plant viruses comprise only a small sampling of Earth's plant virome, since known viruses are mainly those that attract attention due to diseases that they cause on cultivated plants, leaving a large number of viruses that exist in noncultivated plants without causing phenotypic symptoms waiting to be recognized (Fig. 18.1). Traditional detection and diagnostic methods such as host range and indicator host responses, ELISA, electron microscopy (EM), and PCR/RT-PCR remain useful tools for diagnosing known viruses that are suspected of causing diseases, but these methods have shortcomings for discovery of novel viruses. Recent combinations of advanced techniques including viral nucleic acid enrichment methods, purification of viral-like particles from host samples, total RNA or total DNA extractions with sequence-independent amplification, analysis of virus-derived small interference RNAs (vsiRNA) combined with next generation sequencing (NGS), metagenomics or large-scale metatranscriptomic sequencing, and associated bioinformatics methods have resulted in the identification of many novel viruses and viroids (small, circular noncoding RNAs) from samples of all living organisms in land and sea.[247,261,274,320] These novel viruses are filling evolutionary gaps and have greatly expanded our basic knowledge of virology.

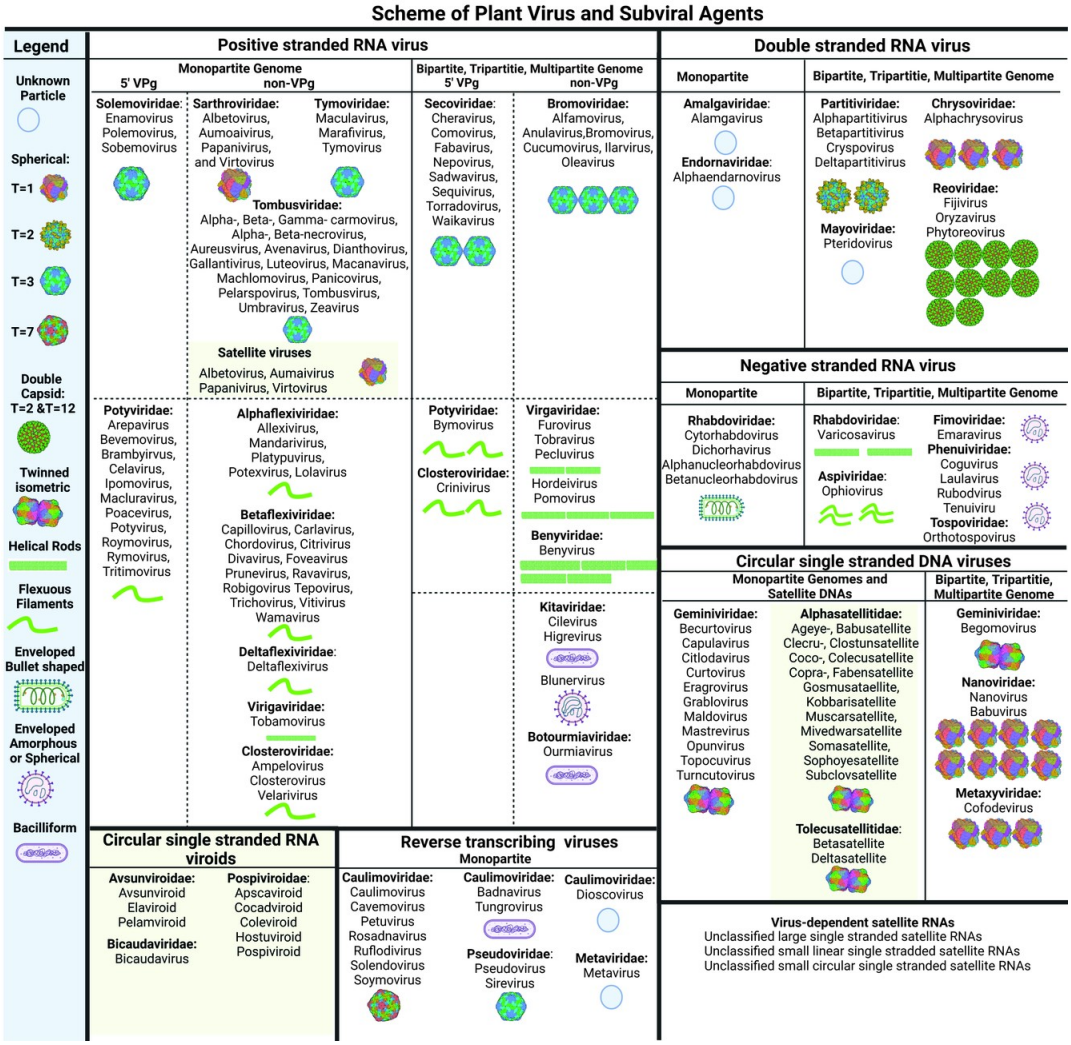

FIGURE 18.1 **Representation of the taxonomic scheme of plant viruses**. The first hierarchy of the scheme identifies RNA or DNA viruses as well as viroids and virus-associated satellites. Positive-strand (+)RNA viruses are separated into columns representing ones that have 5′ VPg and those without VPg. The next hierarchy identifies virus families and genera within those families. The conserved virion structure for each family is represented.

To detect novel plant viruses, plant viral metagenomic approaches have focused on four classes of nucleic acids as targets for virus discovery: (1) total host plant RNA or DNA; (2) virion-associated nucleic acids (VANA); (3) dsRNA replication intermediates or by-products; and (4) vsiRNAs. NGS of total RNA or DNA from a plant sample is the simplest method to explore viral sequences, but viruses represent a very small percentage of total reads. VANA combined with NGS is more laborious, but much more selective and effective, while isolation of dsRNA for metagenomic studies of plant viruses provides an in-depth analysis of virus-specific sequences.[246,247] Infections of both viruses and viroids produce abundant siRNAs covering their entire genome in the plant, with a reduced amount in their insect vectors, making this a very sensitive method of detection.[304] siRNAs are the products of RNA silencing, the host adaptive antiviral response (see section on Host-Virus Interactions) that produces small RNAs from replicating viral RNA genomes or resident stem-loop structures.[321] This method can also detect transcriptionally active integrated endogenous viral elements (EVFs).[247] While these methods have led to the discovery of new viruses and viral nucleic acids, the viruses must be convincingly demonstrated to infect the suspected host plant before a new host-virus combination is confirmed.[321]

Past and present human activities, including expansion of agricultural cultivation and world trading either by companies or individuals receiving uninspected plant materials by post, have affected virus short- and long-distance spread in the environment, host selection and host range, host response and adaptations, and ultimately virus-plant coevolution and codivergence. Studies on the distribution of different isolates of turnip mosaic virus (TuMV; family *Potyviridae*), which mainly infects crops and ornamental plants of the family *Brassicaceae*, illustrates this point. Phylogeographic and molecular clock analyses of over 700 TuMV isolates revealed subgroups with different host ranges spread from the center of emergence to the rest of Eurasia in relation to the host plants grown in each country. Interestingly, the migration pathways of TuMV retraced some of the major historical trade arteries in Eurasia, a network that formed the Silk Road.[126,218,224] Fifteen years of evolutionary analysis also allowed the origin tracing of hop stunt disease, caused by hop stunt viroid (HSVd), which is currently responsible for hop stunt epidemics in commercial hops in Japan, China, and the United States. Over 70% of grapevines were found to be symptomless reservoirs for the viroid, which require few if any mutations to spread and adapt to hops.[113,125,253] Grapevine as a viroid reservoir is illustrated by a very old ungrafted grapevine named “Munage” (King of Grapevine) that has lived in the Xinjiang autonomous region in Northwestern China for more than 150 years (Fig. 18.2). At least three species of viroids coexist in Munage, but still it flourishes.

FIGURE 18.2 **Munage, King of the Grapevine**. The 150-year-old Munage, located in Xinjiang, China, is infected with viroids HSVd, GYSVd-1 (grapeHSVdvine yellow speckle -1), and GYSVd-2 with no visible symptoms. (Reprinted from Jiang DM, Sano T, Tsuji M, et al. Comprehensive diversity analysis of viroids infecting grapevine in China and Japan. *Virus Res*. 2012;169(1):237-245. Copyright © 2012 Elsevier, with permission.)

Current Plant Virus Epidemics

World-wide demand for vegetable and seed markets is expanding with the growth in global populations and the desire of consumers for greater access to food choices. As climate change intensifies, new cropping systems are changing existing farm landscapes and converting natural land into cultivated land, both of which introduce new crops into a shifting geographical environment. Without the benefit of long-term coevolution of crops and pathogens, opportunities are created for disease outbreaks including many caused by viruses.[119] Climate change is also driving the emergence of new virus diseases in wild plant populations that have spilled over into major food crops.[284] The dynamics of global trade and the influence of climate change on temperature and rainfall have impacted the distribution of insects that vector plant viruses, which further threatens food production. Localized and sporadic epidemics are causing hunger in food-insecure regions, while some plant viruses endanger food security on a worldwide scale. Viruses that once existed in distinct landscapes are now sharing hosts with emerging viruses, creating the opportunity for enhanced accumulation and spread, along with altered tissue tropism, expanded host range, vector behavior, and other synergistic interactions that result in devastating diseases.[76,186,199]

Single and complex virus infections are causing major epidemics and pandemics among staple food crops worldwide (Table 18.1). One example is tomato brown rugose fruit tobamovirus (ToBRFV; family *Virgaviridae*), a recently identified virus infecting tomato and pepper that is causing regional outbreaks around the globe. ToBRFV is vectored mechanically by people and equipment and is unaffected by the many known sources of tobamovirus genetic resistance bred into domestic tomato plants.[91] Discovered in Israel in 2014,[177] ToBRFV is a major threat to tomato and pepper production globally, and international phytosanitary standards have been imposed by the International Plant Protection Convention (IPPC) and other trade organizations to ensure safe trade of products between countries.

TABLE 18.1 Examples of current most important epidemic or pandemic plant viruses

Name of Virus or Synergistic Viral Disease	Known or Potential Vectors	Disease Symptoms	Locations and Control Methods
Viruses of cereal crops (reducing crop yield and symptoms affect marketability)			
Maize lethal necrosis disease caused by coinfection of maize with maize chlorotic mottle virus (MCMV; family *Tombusviridae*) and one of several viruses from *Potyviridae* including sugarcane mosaic virus (SCMV), maize dwarf mosaic virus (MDMV), and wheat streak mosaic virus (WSMV)	Thrips (*Frankliniella williamsi*) and beetles transmit MCMV; several aphid species transmit potyviruses	Negligible yield or plant death	Located in Asia, Africa, and the Americas. Chemical control in fields and/or certified seeds that have been tested for known viruses
Maize dwarf mosaic disease caused by maize dwarf mosaic virus (MDMV: family *Potyviridae*)	Several aphid species	Mottling or mosaic leaf symptoms and dwarfing	
Maize streak disease caused by maize streak virus (MSV; family *Geminiviridae*)	Leafhoppers	Yellow or white streak disease on youngest leaves and yield loss	
Maize rough dwarf disease caused by one or a complex of four figiviruses: maize rough dwarf virus (MRDV), Mal de Rio Cuarto virus (MRCV), rice black–streaked dwarf virus (RBSDV), and southern rice black–streaked dwarf virus (SRBSDV)	Planthoppers	Leaves are distorted, panicle discoloration, dwarfing	
Rice tungro disease caused by a complex of rice tungro bacilliform virus (RTBV; genus *Tungrovirus*) and rice tungro spherical virus (RTSV; family *Caulimoviridae*)	Leafhoppers and planthoppers	Leaf discoloration, stunted growth, reduced tillers, sterile or partly filled grains	Tungro disease is located on all continents except Australia, RYMV is specific to Africa, and all the common dwarf diseases are found on all continents. Control by cultural practices and using virus or insect resistant plant varieties
Rice yellow mottle virus (RYMV; family *Solemoviridae*)	Beetles, grasshoppers, and leafhoppers	Leaf streaking and malformation, death of young plants	
Common causes of dwarf or stunted growth diseases in rice are caused by viruses of *Bunyaviridae*, *Rhabdoviridae*, and *Reoviridae*: rice black–streaked dwarf virus (RBSDV), rice dwarf virus (RDV), rice gall dwarf virus (RGDV), rice grassy stunt virus (RGSV), rice stripe mosaic virus (RSMV), rice ragged stunt virus (RBSV), rice stripe virus (RSV), and southern rice black–streaked dwarf virus (SRBSDV)	Leafhoppers and planthoppers	Extreme stunting, leaf discoloration, deformed panicles	
Yellow dwarf and yellow mosaic disease caused by viruses of *Tombusviridae and Potyviridae*: barley yellow dwarf virus (BYDV), cereal yellow dwarf virus (CYDV), and wheat yellow mosaic virus (WYMV)	Several aphid species	Severe yellowing and dwarfing	Located on all continents. Control by cultural practices and chemical control of aphids
Soilborne mosaic diseases in wheat caused by viruses of the family *Virgaviridae*: Chinese wheat mosaic (CWMV) and soilborne wheat mosaic virus (SBWMV)	*Polymyxa graminis*	Mosaic pattern on foliage	Located on all continents. Control by cultural practices
Viruses of horticultural crops (reducing crop yield/marketability and causing tree death)			

Mosaic and necrotic diseases caused by members of the family *Potyviridae*: papaya ringspot virus (PRSV), plum pox virus (PPV), potato virus Y (PVY), zucchini yellow mosaic virus (ZYMV), and turnip mosaic virus	Several aphid species	Mosaic, mottling, ringspot, chlorosis, necrosis patterns on leaves as well as the fruits, tubers, and vegetables reducing or eliminating marketability	Located on all continents with some exceptions. Pepper, lettuce, and tomato infecting viruses are not found in Australia, and CGMMV is not found in South America. Controlled by one or more of the following methods: using insect- or virus-resistant plant varieties, seed certification, cross-protection, and chemical control of insects
Potato leafroll virus (PLRV; family *Solemoviridae*)			
Pepper vein yellows virus (PeVYV; family *Luteoviridae*)			
Pepino mosaic virus (PepMV; family *Flexiviridae*), tobacco mosaic virus (TMV; family *Virgaviridae*), and tomato brown rugose fruit virus (ToBRFV: family *Virgaviridae*)	Unassisted mechanical transmission		
Hot pepper endornavirus (HPEV; family *Endornaviridae*)	Horizontally transmitted		
Tomato yellows diseases caused by members of the genus *Crinivirus*: lettuce chlorosis virus (LCV) and tomato chlorosis virus (ToCV)	Several whitefly species		
Tomato yellow leaf curl virus (TYLCV; family *Geminiviridae*)			
Tomato spotted wilt virus (TSWV; family *Bunyaviridae*)	Western flower thrips (*Frankliniella occidentalis*)		
Beet severe curly top virus (BSCTV; family *Geminiviridae*)	Leafhoppers		
Cucumber green mosaic mottle virus (CGMMV; family *Virgaviridae*)	Unassisted mechanical and seed transmission		
Turnip yellows virus (TuYV; syn beet western yellows virus; family *Luteoviridae*)	Several aphid species		
Cucumber mosaic virus (CMV; family *Bromoviridae*)			
Grapevine leafroll disease caused by several members of the family Closteroviridae: grapevine leafroll–associated virus 1 through 13 (GLRaV) with GLRaV-3 being the most important	Several species of mealybugs and scale insects, grafting of infected grapevine material	Leaf discoloration and downward curling or cupping, delayed and uneven fruit ripening, reduced fruit quality and yield, affects wood causing graft incompatibilities	Located on all continents. Controlled by one or more of the following methods: chemical control of insects, cross protection, culture methods, production of clean propagative wood for planting or grafting, use of certified planting stocks that have been tested for known viruses
Grapevine fanleaf degeneration disease is caused by grapevine fanleaf virus (GFLV: family *Secoviridae*)	Soilborne nematodes	Plants show reduced growth, leaf distortions and unusual chlorosis including yellow mosaic and yellow veins	
Grapevine degenerative decline disease is caused by members of the family *Secoviridae*: tobacco ringspot virus (TRV) and tomato ringspot virus (ToRSV)	Soilborne nematodes	Leaf symptoms include yellow lines and flecks as well as ringspots, vines are unproductive, small grape clusters	
Hop latent stunt viroid (HLVd; family *Pospiviroidae*)	Vegetative propagation	Reduced growth, shorter internodes, stunting	Located on all continents
Cacao swollen shoot virus (CSSV: family *Caulimoviridae*)	Mealybugs	Leaf discoloration, stem/root swelling, die-back, kills trees within a few years	Primarily located in West Africa. Controlled by eradicating trees

Virus species belonging to genera *Begomovirus*,[216] *Tospovirus*,[167,225] *Potyvirus*,[330] *Reovirus*, and *Tenuivirus*[336,341,349,350] represent only a portion of the viruses currently causing devastating diseases. To ensure food safety, new technologies and novel solutions are needed including (1) improved diagnostic testing such as NGS and small RNA (sRNA) sequencing to rapidly identify emerging diseases and improved sanitary certifications[290]; (2) advanced breeding methods including genome editing, which has been successfully used to engineer resistance by targeting viral sequences[6,13] or the plant genome[30,236]; (3) novel sRNA delivery systems (sprays, injections, VIGS) that do not require modification of the plant genome; (4) drone technology in field monitoring; and (5) active communication between growers and researchers and phytosanitary officials who can offer solutions and preventative or eradication measures.

RECOMBINATION IN PLANT VIRUS EVOLUTION

Viruses evolve when genetic and environmental factors lead to increased virus fitness for amplification within a host or transmission between hosts or in acquisition of new hosts. RNA virus evolution is the product of many factors, but most important is the encoded RNA-dependent RNA polymerase (RdRp), which is error-prone and poorly processive. Due to lack of repair functions associated with most RdRp, missense mutations are generated during each replication cycle, generating a population of viral genomes with varying degrees of fitness relative to the

cellular environment. In addition, premature termination by RdRp can lead to RNA recombination, where the RdRp continues synthesis in *cis* elsewhere on the same RNA genome or in *trans* by using host mRNAs or RNA from the same virus species (intraspecies recombination) or different virus species (interspecies recombination), both of which require coinfection of the same cell. Interspecies recombination generates a chimeric, recombinant virus that may have increased fitness due to new combinations of translated products and/or RNA controlling elements.[1,137,197] High mutation rates and low template processivity of RdRp may go hand in hand as generation of deleterious mutations are counterbalanced by high levels of RNA recombination, which rids the viral population of poorly fit variants while enriching viruses containing beneficial mutations to speed adaptation to selective pressure from environmental variations.[325] Recombination can also generate replication-competent subviral RNAs, such as defective interfering RNAs and satellite RNAs, whose properties can be beneficial for viral infection.[266] Along with polymerase-driven recombination, nonreplicative recombination also occurs, but at a much lower frequency using poorly understood processes that require one or more internal RNA lesion and subsequent ligation of RNA segments.[137] Other viral-encoded proteins can also affect recombination, as evidenced by mutations generated in CP-binding RNA motifs significantly reducing recombination in brome mosaic virus (BMV; family *Bromoviridae*).[275] Host factors can also play a role in recombination, with different BMV recombination "hot-spots" found in different hosts,[138] and at different times in the infection of a host by turnip crinkle virus (TCV: family *Tombusviridae*).[27] Forty host proteins affecting tombusvirus recombination were identified using a yeast temperature-sensitive library that included several actin network proteins that reduced recombination and a cellular DEAD-box helicase that enhanced recombination.[234] In addition, generation of replication-competent recombinants between insect and plant viruses in yeast and plants was positively affected by reduction of the cellular SERCA-like Pmr1 ATPase-driven Ca^{2+}/Mn^{2+} pump, which regulates intracellular Ca^{2+} and Mn^{2+} levels in the Golgi apparatus.[146]

Recombination in the *Potyviridae*

Potyviridae, the largest family of plant viruses, has members that contain a single stranded positive sense (+)RNA genome with a 5′ VpG and a 3′ polyA tail. Potyvirids encode a single polyprotein that is proteolytically processed into 10 mature proteins and two additional extension products. Although potyvirids only infect plants, they contain polyphyletic genomes with three proteins showing significant similarities to proteins from three unrelated viruses: (1) the DEAD helicase-helicase C region of the C1 protein, which is related to that of classical swine fever and hog cholera pestiviruses; (2) the CP, with its core domain rich in alpha helices, is structurally similar to that of orthomyxoviruses and bunyavirids; and (3) the RdRp, which shares significant similarity with those of animal and avian astroviruses.[78] Since no other sequence relationships are discernable between potyvirids and these or any other virus, recombination in a common ancestral host was likely an important factor in the evolution of the modern *Potyviridae*.

Phylogenic studies to identify common ancestors among the 12 *Potyviridae* genera identified discrete lineages based on the assumption that divergence results from accumulating mutations from common progenitor viruses. Evidence indicates that hosts (monocot or dicot) and vector niches are important contributors to their evolutionary divergence.[135] The central role of recombination in the diversification of potyviruses has also been well established,[22,180,277] with intraspecies recombination being common and interspecies recombination appearing to be quite rare.[78,229] Using viruses with different markers in their genomes to identify recombinants, and assuming that trans recombination only occurs in coinfected cells, Tromas et al.[285] estimated a very high rate of recombination, 3.3×10^{5} recombination events per nucleotide site per generation, which is similar to the potyvirid rate for individual mutations. However, this exceptionally high value was based on reports suggesting very low levels of cellular coinfection by potyvirus strains.[47] This view was recently challenged by a report examining epidermal cells in two different hosts, where many cells were found to be coinfected by two distinct potato virus Y (PVY) strains, with the amount of colocalization changing over the course of the infection.[198] The exceptionally high level of potyvirus recombination that likely results from prevalent cell coinfection, along with other factors, has contributed to the emergence of numerous recombinant strains in the United States and Europe that are transmitted more effectively by their aphid vectors compared with the single dominant strain that was present in the last century, causing significant problems currently for the potato industry.[81]

Recombination in the *Tombusviridae*: Umbraviruslike RNAs

Umbraviruses (family *Tombusviridae*) are positive sense (+)RNA viruses that lack an encoded CP and thus depend on a coinfecting virus member of the family *Solemoviridae* for encapsidation. As with all members of the *Tombusviridae*, umbraviruses encode a replication-required protein and an extended version of this protein that is generated by -1 ribosomal frameshifting, which is the viral RdRp (Fig. 18.3A). In addition, umbraviruses code for two proteins that allow for cell-to-cell and long-distance vascular movement, and the assisting virus benefits from the umbravirus cell-to-cell movement protein to escape from vascular cells.[276] Recently, subviral (+)RNAs that encode an RdRp with significant similarity to umbraviruses also generated through a −1 ribosomal frameshifting were identified by NGS performed across several monocot and dicot plants.[170,306] These umbravirus-like RNAs (ulaRNAs), which contain umbravirus-related 3′ terminal sequences and structures as well as umbravirus-like transcription start sites, were divided into three classes based on maximum likelihood phylogenetic analyses (Fig. 18.3C) and additional secondary structure similarities.[170] Class 1 ulaRNAs only encode the two replication-required proteins and are found in papaya and babaco (mountain papaya) in Central and South America. All class 2 ulaRNA (with one exception) have at least one additional ORF (ORF5) that overlaps with the end of the RdRp ORF in the −1 frame, and class 2 infecting monocots have an additional ORF (ORF6) in the −2 frame that is nearly completely imbedded within ORF5 (Fig. 18.3B). The function of the ORF5/ORF6 products are not known, but the ORF5 amino acid sequence contains motifs found in the 30 kDa superfamily of plant virus movement proteins.[170,207] Class 2 ulaRNA citrus yellow vein–associated virus (CYVaV-citrus) has no additional ORFs due to (1) mutations near the presumptive subgenomic RNA transcription start site; (2) mutations at the translation start site; and (3) two extensive deletions within the analogous ORF5 region (Fig. 18.3D and E). Class 3 ulaRNAs have one of two different ORFs of unknown function that are not related to ORFs 5 or 6 and the RdRp overlap region is different (Fig. 18.3B).

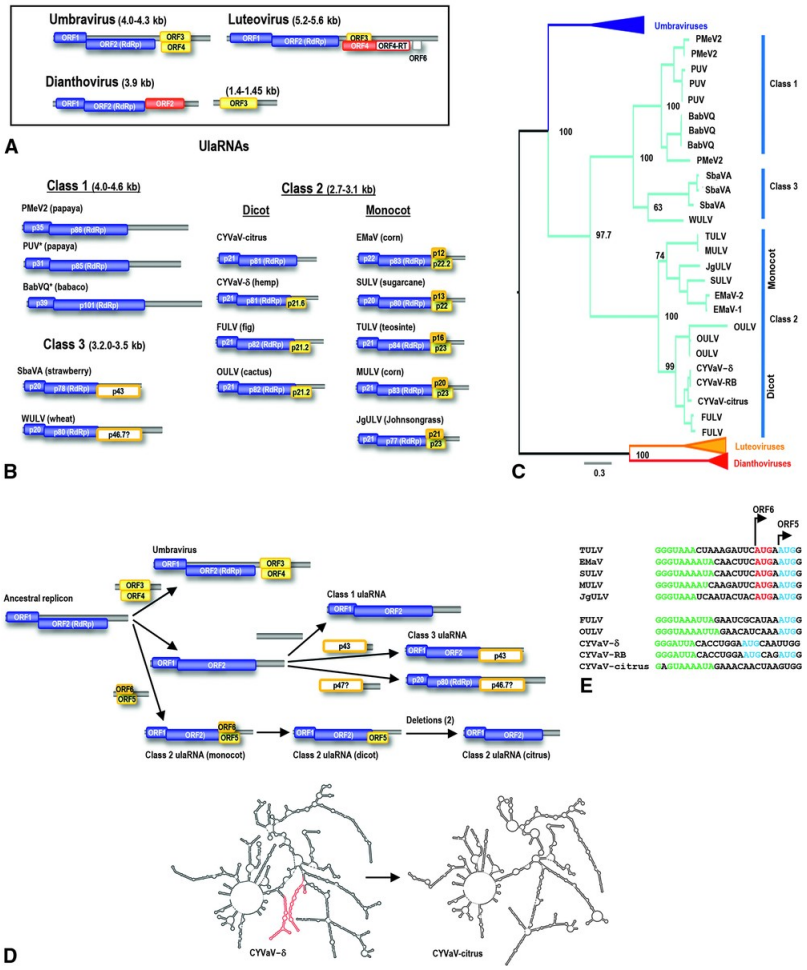

FIGURE 18.3 **Recombination in the evolution of umbraviruslike (ula)RNAs**. **A**. Gene organization of umbraviruses and two other genera of umbraviruses and viruses from two other genera of family Tombusviridae that generate their RdRp through ribosome -1 frameshifting. Replication-required proteins (ORFs 1 and 2) are in *blue*, MPs are in *yellow*, and CPs are in *orange*. **B**. The three classes of ulaRNAs. Class 1 have extensive 3′ UTRs; class 2 ulaRNAs (with the exception of CYVaV-citrus) have an additional ORF (ORF5) with motifs characteristic of the 30 kDa class of MPs. Monocot-infecting class 2 ulaRNAs have an embedded ORF (ORF6) of unknown function. CYVaV-citrus has two large deletions (see **D**, *bottom*) and other changes (see **E**) that eliminate ORF5. Class 3 ulaRNAs form a monophyletic group based on the RdRp sequence but contain additional ORFs of different origin. **C**. Phylogenetic tree based on RdRp amino acid sequences show ulaRNAs and umbraviruses share a common ancestor. Tree was midpoint rooted. **D**. Model for the origin of ulaRNAs. The model suggests that multiple recombination events led to the different classes of ulaRNAs. (*Lower*) The secondary structure of CYVaV-citrus and the proposed secondary structure of CYVaV-δ are shown. Sequence missing in CYVaV-citrus is in *red*. **E**. Sequence at the initiation sites of ORF5 and ORF6. An umbravirus-like sgRNA promoter is just upstream of this sequence, which begins with a sequence (in *green*) that is commonly found at the 5′ ends of gRNAs and sgRNAs in umbraviruses and carmoviruses. ORF6 initiation codon is in *red*, and ORF5 initiation codon is in *blue*. (Phylogenetic tree courtesy of Sayanta Bera.)

Based on related RdRp and conserved 5′ and 3′ sequences and structures, a model was proposed suggesting that the progenitor for both umbraviruses and ulaRNAs was a class 1-type ulaRNA, with different recombination events introducing different putative MP-encoding genes or noncoding sequences into the viral genomes (Fig. 18.3D).[170] The recombination event that introduced ORF5/6 also included an efficient 3′ cap-independent translation enhancer (3′CITE)[171] (see section on (+)RNA Virus Translation below) not found in class 1 and class 3 ulaRNAs. Unexpectedly, several class 1 and class 2 ulaRNAs were reported in infected plants in the absence of any detectable helper virus.[151,170,306] One of these was CYVaV- citrus, the class 2 member without ORF5. CYVaV has only been found once, in four southern California limequat trees with yellow vein symptoms in the 1950s.[307] When trees containing the original grafted materials were examined many years later by NGS, no helper virus was found.[151] This suggest that at least some ulaRNAs may be capable of an independent existence in plants, even in the absence of any additional ORFs, which was confirmed for CYVaV-citrus, CYVaV-δ, and opuntia umbralike virus (OULV) (Simon, unpublished). What advantage CYVaV-citrus might have acquired by losing ORF5 is not known.

Geminivirus, Potyvirus, and Negative-Strand Virus Infection Cycles

Plant viruses initiate infections following assisted or unassisted entry into the first cell(s). Assisted transmission requires vectoring by mites, insects, parasitic plants, humans, protists, or nematodes, whereas unassisted transmission includes pollen and seed transmission, contacts between foliage, roots, other plant parts, as well as via water, exudates, and soil. Unlike animal viruses, cell entry is never through interaction with cell surface receptors but rather requires processes that either disrupt the cellulose wall surrounding cells or seed/pollen transmission. The cues for virion disassembly are largely unknown but can be coupled to translation of the exposing genome for (+)RNA viruses. The genomes of (−)RNA viruses need to be transcribed to (+)RNA by the encapsidated viral RdRp prior to protein synthesis. In the presence of RdRp and other virus-encoded replication proteins, modifications of host endomembranes occur leading to the formation of clustered replication organelles (see

section on (+)RNA replication). The genomes of double-stranded (ds)RNA viruses cannot be uncoated to avoid activation of antiviral RNA silencing, which targets dsRNA. Instead, the dsRNA genome segments become transcribed within the particle, after which the capped transcripts are transported into the cytoplasm. The genomes of single-stranded (ss)DNA and dsDNA viruses replicate and become transcribed in the nucleus and must modify nuclear processes to ensure successful multiplication.

Plant viruses employ various genome expression strategies to provide temporal and quantitative control over expression of viral proteins needed for replication, encapsidation, cell-to-cell and systemic movement, and vector transmission. Common strategies are division of ORFs on separate genomic (g)RNAs, synthesis of polyproteins, ribosome recoding, and/or the production of subgenomic RNAs (sgRNAs). sgRNAs are frequently the templates for expression of proteins needed for genome encapsidation, as expression is delayed in comparison to gene expression for the gRNA, which ensures that replication is not interrupted by premature particle assembly. Cellular defense measures elicit viral counter measures that include suppression of RNA silencing and nonsense-mediated decay (NMD) (see section on virus-host interactions) and ensures translation of sufficient proteins for encapsidation and transmission to the next plant.

Plant viruses transit out of infected cells through structures called plasmodesmata (PD). PDs provide cytoplasmic continuity between adjacent cells, facilitating diffusion as well as active transport of molecules, proteins, and all types of cellular RNAs. Plant viruses evolved specialized movement proteins (MPs) and several strategies for cell-to-cell movement through PD (see section on virus movement). Systemic spread of the infection to the entire plant requires delivery of viral genomes into the phloem (part of the plant's vascular system) and from the phloem to cells in systemic leaves and roots. Viral genomes require protection during movement, which unlike animal viruses can involve (depending on the virus) virions or complexes composed of gRNA, MP, and additional proteins (vRNPs).

Until recently, replication, translation, and the spread of viruses in plants were considered relatively simple processes. However, with the development of more sophisticated research tools, the astonishing complexity of these processes has become apparent, including the involvement of hundreds of host proteins that both assist and counter viral infections. Here, we summarize some characteristics of the infection cycles of gemini- [ssDNA]), poty- [(+)RNA], and tospo- [(−)RNA] viruses to exemplify recent advances, followed by sections where we delve more deeply into current knowledge on selected host-virus interactions associated with replication, translation, movement, and host defense.

Geminiviruses

Family *Geminiviridae* currently consists of 14 genera and 520 species. Geminiviruses comprise a major part of the plant virome[246] and have either mono- or bipartite circular ssDNA genomes that total 2.5-5.2 kb. Depending on the genus to which they belong, geminiviruses are vectored by phloem-feeding whiteflies, leafhoppers, or treehoppers, and virus infection is often limited to the phloem. Although active nuclear DNA synthesis is required for geminivirus replication, these viruses typically infect terminally differentiated cells that lack active DNA synthesis machinery. Geminiviruses encode proteins that activate the cell cycle so that cells enter S-phase, while also interfering with host transcription, cellular homeostasis, and developmental processes to create a cellular environment that favors virus infection.[3,92] Monopartite begomoviruses are frequently associated with β-satellites that have ssDNA genomes unrelated to the helper geminivirus genome and encode a protein with an important role in symptom induction, suppression of transcriptional and post-transcriptional gene silencing, and regulation of MAP kinase pathways.[100,353]

Upon entry of the ssDNA genome into the plant cell nucleus, the viral ssDNA genome is replicated by host DNA polymerase α and δ through a dsDNA intermediate by rolling circle replication. Recombination-dependent replication is facilitated by the multifunctional viral-encoded Rep initiator protein, which cleaves the geminiviral genome at a conserved sequence within an intergenic region to initiate replication[141,319] (Fig. 18.4). The replication ancillary protein C3 of curto- and begomoviruses enhances replication by fine-tuning the recruitment of DNA polymerase subunit δ (in preference to subunit ε), to synthesize ssDNA molecules.[295] Rep restores the circular ssDNA genome structure and releases the nascent ssDNA molecules. Partially replicated ssDNA can recombine with viral dsDNA to ensure reliable replication. Newly formed ssDNA molecules are packaged into geminate particles via their interaction with CPs after which the virus is transmissible by the biological vector.

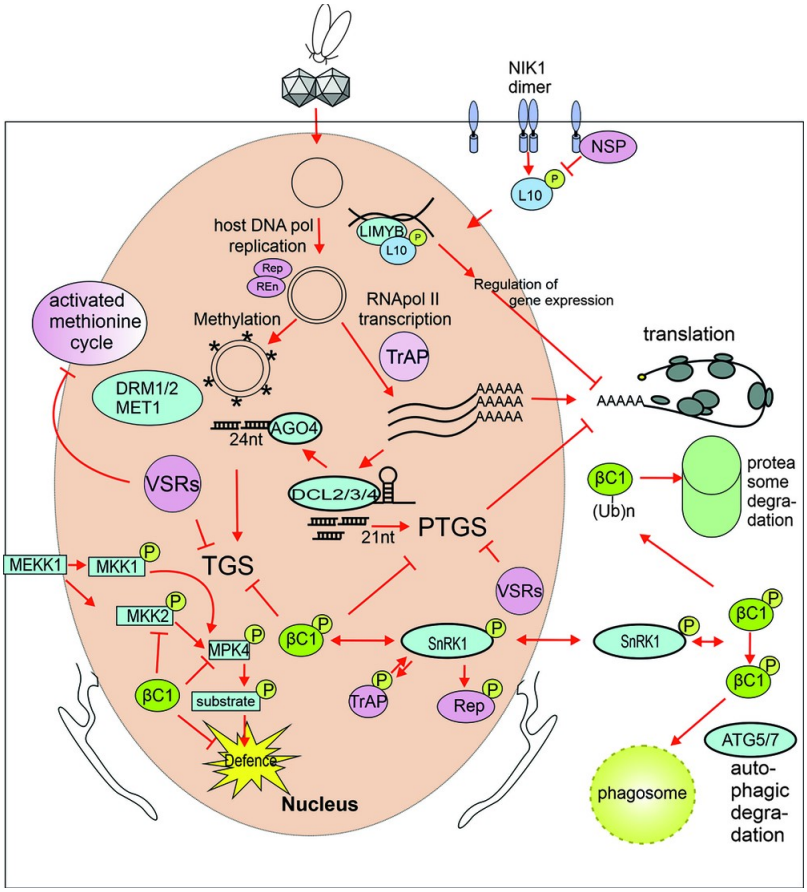

FIGURE 18.4 **Geminivirus infection cycle and interactions with the host cell**. Geminiviruses enter the cell via vector-assisted transmission. The uncoated ssDNA genome is transported to the nucleus of the cell, where it is amplified by host DNA polymerase via a dsDNA intermediate. Geminivirus bidirectional dsDNA transcription that is catalyzed by host RNA pol II produces viral mRNAs that are translated into viral proteins on host ribosomes. The replication initiator protein Rep initiates rolling circle replication by creating a nick into a viral dsDNA molecule to generate a primer for ssDNA synthesis. Replication enhancer protein REn contributes to efficient genome amplification. The host cell fights the infection with different responses. RNA silencing pathways, both transcriptional gene silencing (TGS) that operates with 24 nt sRNAs and transcriptional gene silencing (TGS) with 21 nt sRNAs, are activated upon geminivirus infection. Geminiviral suppressors of RNA silencing (VSRs) inhibit the antiviral activity of TGS and PTGS by (1) interfering with host cell metabolic pathways and key enzymes and (2) interactions with activated methionine cycle enzymes, which are essential for methylation of geminiviral dsDNA by DRM1/2 and MET1 methyltransferases during TGS. Many geminiviral proteins are substrates of kinase complex SnRK1 that regulates energy homeostasis of the host cell. Both proteasomal and autophagic degradation machineries contribute to the outcome of geminivirus infection, thereby degrading post-translationally modified βC1 protein derived from ssDNA βsatellite that associates with diverse geminiviruses. The βC1 protein is involved in the inhibition of the defense response activated by the MAPK pathway. Geminivirus infection detected by PM receptorlike kinases triggers an immune response that, in the case of the NIK1 receptor, results in translation inhibition. Details of the geminivirus infection cycle including its cell-to-cell and long-distance transport and cellular responses that are triggered are presented in the corresponding parts of the text. Viral proteins are shown in *pink*, βsatellite is in *green*, and host proteins that either promote or prevent infection on a *blue-gray* background. (Modified from Gupta N, Reddy K, Bhattacharyya D, et al. Plant responses to geminivirus infection: guardians of the plant immunity. *Virol J*. 2021;18:143. http://creativecommons.org/licenses/by/4.0/.)

Host RNA polymerase II transcribes the geminivirus dsDNA, enabling the translation of C1/Rep and other viral proteins. The geminiviral AC2/C2, also called TrAP, serves as the transcriptional regulator for late gene expression and contributes to the suppression of host defenses.[82] AC2 stimulates transcription of the late gene encoding nuclear shuttle protein BV1 through an abscisic acid (ABA) responsive promoter element.[272] To counter antiviral defenses on an epigenetic level, TrAP serves as a H3K9me2 histone methyltransferase activity inhibitor, preventing the H3K9me2 factor from acting on the viral mini-chromosomes to block gene expression through methylation-dependent restriction.[29]

Potyviruses

Family *Potyviridae* consists of twelve genera, with members containing (+)RNA genomes of 8.2-11.5 kb and flexible nonenveloped filamentous particles with modal lengths of 650-950 nm and widths of 11-20 nm. Potyvirus is the largest genus with 190 current member species. Potyviruses infect cultivated plants worldwide and are abundantly present in wild plants.[246] The infection cycle of potyviruses was recently reviewed[330] and is schematically depicted in Figure 18.5. As described above, VPg is covalently linked to the 5′-terminal nucleotide of the genomic RNA, and the 3′ end is polyadenylated. The genome encodes a major polyprotein that is co- and post-translationally processed by three viral-encoded proteinases into 10 mature proteins, and an 11th trans-frame protein P3N-PIPO, which is produced from two ORFs fused together due to transcriptional slippage that occurs on a fraction of gRNAs.[64,195] The genome organization of potyviruses has similarities to animal picornaviruses including VPg-linked gRNA and similar order of cistrons encoding for the replication proteins in the polyprotein

encoding ORF. The potyvirus core replicase proteins are named as follows: the viral RNA helicase is cylindrical inclusion protein CI, the membrane spanning protein is 6K2, VPg, a chymotrypsin-like cysteine protease related to the picornavirus 3C protease is nuclear inclusion protein a–protease (NIa-Pro), and nuclear inclusion protein b (NIb) is the RdRp.

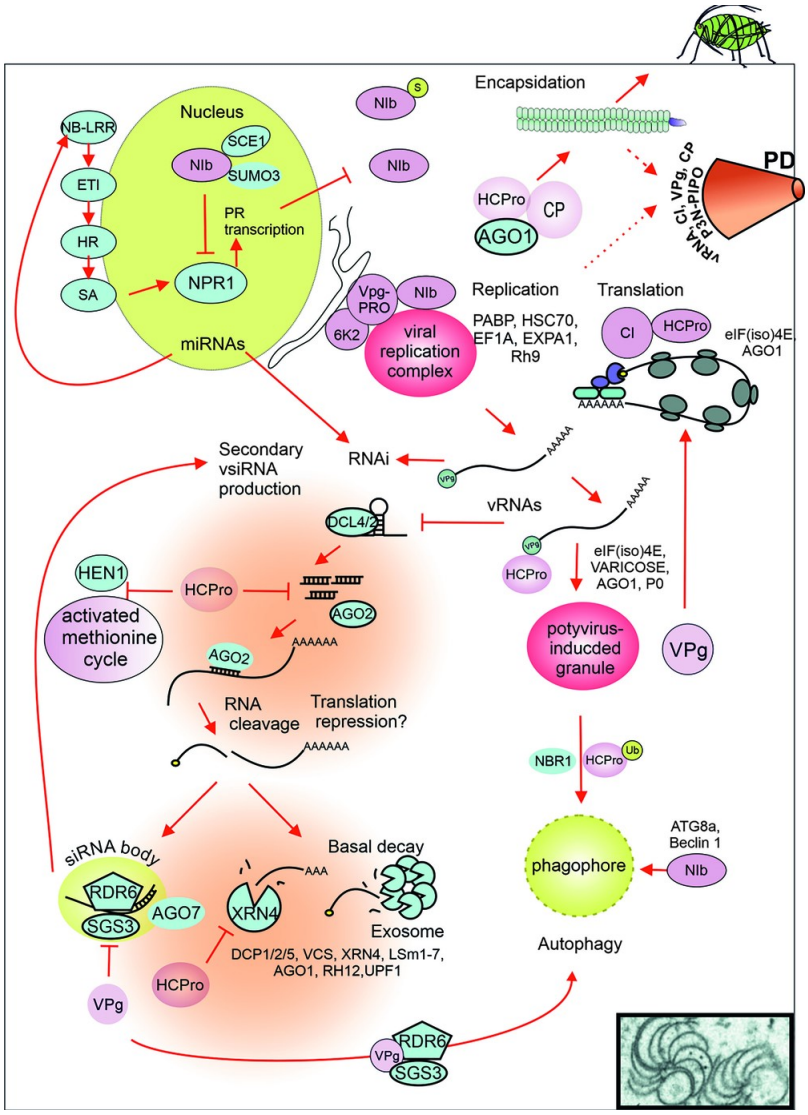

FIGURE 18.5 **Potyvirus infection cycle and interaction with the host cell**. After entering the cell with the help of an aphid vector and uncoating, the potyviral ssRNA genome initiates synthesis of viral proteins and formation of the viral replication complex in association with the ER. Potyvirus infection activates RNA silencing (RNAi), during which host proteins DCL4/2 and AGO2 are key enzymes. When RNA silencing suppression fails, viral RNAs are cleaved and intact viral RNAs serve as the templates for formation of secondary vsiRNAs to amplify RNA silencing responses and interfere with host cell gene expression. The RNA silencing pathway and 5′-3′ decay pathway of mRNAs interact during potyvirus infection. Potyviral VSRs, HC-Pro, and VPg serve in diverse functions in RNAi suppression to promote infection by interfering with accumulation of both siRNAs and secondary vsiRNAs. These proteins escort the replicated viral RNA to ribosomes for viral protein synthesis together with several host proteins through a pathway that includes formation of potyvirus-induced granules. Silencing suppression-associated granules are targeted by autophagy as a second layer of defense, which also includes the RDR6/SGS3/VPg protein complex required for vsiRNA production. Recognition of potyvirus infection by an R-gene activates an NPR1-mediated immune response, whereas potyviral RNA polymerase NIb suppresses this response via its interactions with the host SUMOlylation pathway. Host miRNAs interfere with potyvirus immune responses. The intracellular trafficking pathway to complete potyviral RNA amplification, cell-to-cell and long-distance RNA transport, and many details of immune responses are described in the corresponding parts of the text. Encapsidation of potyvirus genome into stabile particles require both viral and host proteins, such as AGO1. Potyvirus cell-to-cell movement via plasmodesmata (PD) requires the conical structures formed by CI protein and several viral and host proteins. Later in the course of infection, the conical structures detach from the PD and can be detected as hallmark structures of potyviruses (**inset**) by electron microscopy. Host proteins are in *blue*; viral proteins are in *pink* and *orange*.

Viral RNA translation initiates on rough ER, and potyviral 6K2 or 6K2-VPg-NIa-Pro precursor proteins assist COPI- and COPII-dependent virus replication complex formation at ER exit sites.[115,309] Most potyviral proteins, excluding P1, P3N-PIPO, and likely CP, and many host proteins like GADPH, eEF1A, HSPs 70 and 90 are present in the potato virus A (PVA) replication complex.[172] During TuMV infection, 6K2 is essential for release of motile replication vesicles from the ER. 6K2 engages host RHD3, an ER fusogen, and NIb recruits NbEXPA1, a host α-expansin, to promote TuMV replication and cell-to-cell movement.[202,227] Together with prevacuolar SNARE protein VTI11, 6K2 directs TuMV replication vesicles to an unconventional Golgi apparatus bypass pathway.[24] Dynaminlike proteins AtDRP1A and AtDRP2 required for endosomal trafficking promote TuMV infection and interact with several TuMV replication and cell-to-cell movement proteins, indicating that potyviruses may also recruit late endosomes for replication.[316,322] The SNARE protein Syp71 mediates the chloroplast fusion of TuMV replication vesicles

to ensure robust replication.[310] In the PVA replication complex, 25% of the proteins are derived from chloroplasts.

The close association of replication and translation is demonstrated by the specific presence of most ribosomal proteins in the PVA replication structures.[172] Current data indicate that production of potyviral proteins is highly dependent on VPg, especially interaction of VPg with eukaryotic initiation factor 4E (eIF4E) and eIF(iso)4E, but also many other viral and host proteins.[88] Potyviral HC-Pro has an important postreplication role in assisting the VPg-linked viral RNA to the RNA silencing suppression pathway, which includes formation of potyvirus-induced granules (PGs; Fig. 18.5). Many of those host proteins that enhance potyviral translation in association with VPg, such as ribosomal P0 protein, VARICOSE, and AGO1, also localize to the microscopically visible PGs.[43,88,232]

Spread of infection to adjacent healthy cells and systemically to other parts of the plant requires the coordinated action of many potyviral proteins including HC-Pro, P3N-PIPO, CI, 6K2, VPg, and CP.[241] CI forms a conical structure that anchors to PD to assist potyviral RNA transport through the channel (Fig. 18.5). Later, this cylindrical inclusion structure detaches from the PD and exists as a pinwheel-like structure in the cytoplasm, which is an indicator of potyvirus infection. Interactions between the potyviral P3N-PIPO and the host PCaP1 protein enable localization of the movement complex to the PD.[298] A developmentally regulated plasma membrane (PM) protein of *Nicotiana benthamiana* (NbDREPP) interacts with P3N and CI and influences cell-to-cell movement.[72] The majority of amplified (+)RNAs are encapsidated, and studies show that viral replication and particle formation are tightly linked.[68,288]

Negative-Strand RNA Viruses

The phylum *Negarnaviricota* was established in 2019 by the International Committee on Taxonomy of viruses (ICTV) to encompass all negative-sense (−)RNA viruses. The monopartite viruses of the order *Mononegavirales* include six genera, a few of which infect plants and are naturally transmitted by arthropods (or plasmodiophorid protists in the case of lettuce big-vein associated varicosavirus). The enveloped virions contain the gRNA encapsidated by nucleocapsid (N) protein and the RdRp. The order *Serpentovirales* are segmented filamentous viruses and the genus *Ophiovirus* includes plant-infecting members that are also transmitted by plasmodiophorid protists. Additional enveloped plant viruses with three or more genome segments are assigned to the order *Bunyavirales* and the family *Fimoviridae*, genus *Emaravirus*; the family *Tospoviridae*, genus Orthotospovirus; and the family *Phenuiviridae*, genus *Tenuivirus* and genus *Coguvirus*.[23,142] These virions also contain the viral RdRp and nucleocapsid surrounding the RNA genome. Fundamental research on these plant viruses has been recently accelerated with the development of reverse genetic systems for sonchus yellow net nucleorhabdovirus (family *Rhabdoviridae*), tomato spotted wilt tospovirus (TSWV; family *Tospoviridae*), rice stripe tenuivirus (RSV; family *Phenuiviridae*), and rose rosette emaravirus (RRV; family *Fimoviridae*).[57,58,111,295] A yeast replicon system for TSWV has also been reported.[108] The plant infecting members of *Bunyavirales* has been extensively reviewed.[143] The oldest and most well-known member is TSWV, which has become one of the most important and devastating plant viruses worldwide. TSWV is vectored by *Frankliniella occidentalis*, the Western flower thrips.[143]

Within the order *Bunyavirales*, all viruses appear to have gRNA with complementary 3′ and 5′ ends of the genome that form a dsRNA panhandle to create a pseudo-circular appearance during replication. Studies in yeast showed that the terminal 15-17 nt at the 5′ and 3′ ends are essential for activation of the RdRp[108] and likely act as promoters for replication through a viral complementary (vc)RNA. The tospoviruses have three genome segments denoted as large (L, 9 kb), medium (M, 4.8 kb), and small (S, 2.9 kb). The (−)RNA serves as a template for mRNA transcription and viral mRNAs are produced by cap-snatching, whereby the 5′ 12-8 nt of a host mRNA, including the methylated guanosine cap, are cleaved by viral endonuclease activity and used to prime transcription on the viral RNA template. Thus, viral mRNAs differ from the antigenomic RNA molecules that are required for replication due to the presence of a heterogeneous nonviral sequence at the 5′ end. The mRNAs encode (in the positive polarity) the RdRp from the L segment, the envelope glycoprotein (G) from the M segment, and the nucleocapsid (N) protein from the S segment. In opposite orientation, the M segment also encodes the nonstructural NSm protein, which is the viral MP, and S segment encodes NSs, which is a silencing suppressor protein. NSm produces tubular structures that extend between cells for movement of the viral nucleoprotein complex involving the N protein.

The functional domains of the bunyavirus RdRp are also well conserved across families of viruses infecting a wide range of hosts.[9,62,278] For TSWV, the N-terminal endonuclease domain is preceded by an N-terminal extension of unknown function. Following the endonuclease region are eight conserved motifs (A-H), which provide viral RNA binding and polymerase activities. The crystal structure of the TSWV N protein shows that it has structural homology with other orthobunyaviruses and some phleboviruses and forms a trimer subunit surrounding the viral RNA.[84] The replication cycle for (−)RNA viruses is poorly understood. However, given the extensive conservation of RdRp and N proteins, (−)RNA likely enters the cytoplasm as ribonucleoprotein segments that are transcribed by virion-associated RdRp activity. The positive-sense mRNAs derive in part from scavenged host cell mRNAs (via cap snatching) and may be devoid of mRNA at their 3′ ends. Following translation, the viral RdRp is available for further rounds of mRNA synthesis as well as for genome replication through the synthesis of full length complementary RNAs. Virions form by budding through the Golgi apparatus.

Arabidopsis mutant lines with defects in the RNA decay machinery were inoculated with TSWV to identify host genes that can influence virus infection. TSWV infection in plants with mutations in either decapping 2 (DCP2) and exoribonuclease 4 (XRN4), which are engaged in decapping and degrading mRNAs via NMD, or mutations in the Dicer-like proteins (DCL) that are essential for RNA silencing and can attack viral RNAs, revealed that DCP2 and XRN4 played greater roles in TSWV RNA degradation than DCL proteins. This points to the importance of the decapping of cellular RNAs and the availability of caps for TSWV infection.[179] Other host factors critical for virus amplification include the eukaryotic translation elongation factor 1a (eEF1A) and ribosomal protein S6. It is likely that these proteins have features beyond their roles in global mRNA translation that can be hijacked by TSWV, although their direct mechanistic contributions to virus replication are not known.[95,96,139]

(+)RNA Virus Translation

Because of space limitations, this section is confined to a discussion of recent advancements in cap-independent translation of (+)RNA viruses, which are the most thoroughly investigated.

Cap-Independent Translation: Internal Ribosome Entry Sites

Translation of (+)RNA viruses begins shortly after virions enter a new cell and release of the encapsidated (+)RNA genome, or upon entry of a vRNP complex. Like animal viruses, plant (+)RNA viruses have a variety of 5′ and 3′ ends that dictate the mode of translation. Viral genomes that contain the canonical 5′m7GpppG cap attract translation factors and ribosomes similar to host mRNAs, whereas genomes with covalently linked 5′ VPgs or free 5′ phosphates attract ribosomes using alternative sequences and/or structures. The expansive internal ribosome entry sites (IRES) associated with noncanonical translation of 5′ terminal or internal ORFs of animal viruses are largely missing from plant viruses. Known or putative IRES, when they exist, are mainly unstructured sequences of 50-150 nt that can be pyrimidine- or purine-rich[73,196,267] (see Fig. 18.6C). For example, internal translation initiation of the capsid protein ORF in TCV (family *Tombusviridae*), which is likely required early in the infection of the cell for its RNA silencing suppressor function, requires an A-rich sequence that can be functionally replaced with the reverse or reverse complement, and translation is stimulated *in vitro* by addition of eukaryotic translation initiation factor 4G (eIF4G). In addition, mutations outside of the IRES that allow downstream sequences to pair with IRES sequences to form a hairpin structure strongly reduced activity.[189] Weakly structured IRES were reported at the 5′ ends of several VPg-containing potyviruses,[196] with the best studied being the AU-rich pseudoknot in the tobacco etch virus 5′ UTR that binds to eIF4G[239,340] and is frequently used to drive internal translation in virus vectors. Nepoviruses blackcurrent revision virus (BRV) and tomato ringspot virus (ToRSV; family *Secoviridae*) also contain a 5′ VPg but have more structured 5′ IRES elements with multiple poly-pyrimidine stretches in unpaired loops and require uncharacterized 3′ CITEs (see section Cap-independent translation enhancers) for function.[122,123,228] Unlike other potyviruses, the triticum mosaic virus (TriMV) IRES contained within an expansive 739 nt 5′ UTR is more similar to picornavirus IRES and directs translation to the 13th AUG.[244] As with picornavirus IRES, the TriMV IRES binds to eIF4G[243] and contains a 16 nt polypyrimidine segment 11 nt upstream from the translation start site.[112] Another potyvirid with an unusual 5′ IRES is the bipartite wheat yellow mosaic virus (WYMV).[74] Two weak hairpins in the 5′ UTR of RNA1 each contain two residues that interact with identical bases in the sequence between the two hairpins, suggesting that dynamic exchanges of base-pairing are necessary for efficient IRES activity. Sequences within the WYMV IRES interact with eukaryotic translation initiation factor 4E (eIF4E) and connect with the 3′ UTR through long-distance base-pairing.

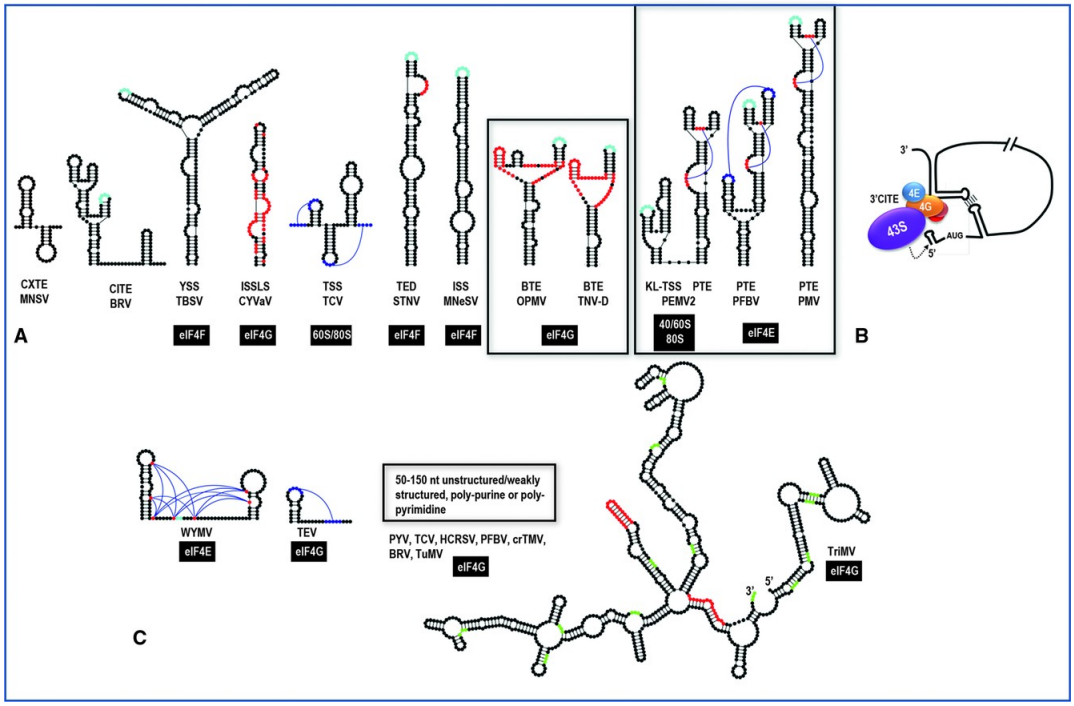

FIGURE 18.6 **Structures involved in cap-independent translation**. **A**.3′ end proximal cap-independent translation enhancers (3′ CITEs). Names of the CITEs are shown above the virus names. Bases in *red* are highly conserved among related viruses. Bases in *light blue* are involved in long-distance interactions with the 5′ end. Bases in *dark blue* and/or *dark blue lines* represent known local tertiary interactions. Translation factors that directly bind the elements (if known) are boxed in *black*. Multiple versions of the same element are boxed. **B**. Schematic representation of the long-distance interactions connecting the 5′ end with 3′ CITEs and assembled translation initiation complex. **C**. Plant virus IRES. Coloring is as in **(A)** except for the TriMV IRES, where AUG sequences are in *green* and *red* sequences denote critical elements required for IRES function.

Cap-Independent Translation Enhancers

The best studied translation elements in plant viruses are the 3′ proximal CITEs (Fig. 18.6A). Viruses can contain one or multiple 3′CITEs, with the three 3′CITEs of PEMV2 used differentially for translation of the gRNA and the sgRNA.[69] Previous reviews of 3′CITEs identified about 7 types that are grouped according to their secondary structures and abilities to bind to particular translation components, usually eukaryotic translation initiation factor 4F (eIF4F) through either its eIF4E or eIF4G subunits, or directly to ribosomal subunits.[196,265,286] However, it is now recognized that many viruses harbor 3′CITEs with structures and features outside of these groupings,[170,228] most of which remain to be characterized. In addition, not all 3′CITEs of a certain type share conserved features, as demonstrated by panicum mosaic virus (family *Tombusviridae*) translation enhancers (PTEs), some of which contain an additional hairpin and kissing-loop interaction,[118] and barley yellow dwarf virus (BYDV; family *Solemoviridae*) translation enhancers (BTEs), which contain at least three different sub-types that share signature features but also contain their own distinctive conserved sequences[106] (Fig. 18.6A). Interestingly, virus members of the same genus can share highly conserved structures at their 3′ termini and have different 3′CITEs just upstream, suggesting that recombination has played an important role in the addition of 3′CITEs to plant virus genomes.[265]

Several 3′CITEs have been shown to assemble translation complexes, including 40S subunits, in the 3′ region of viral genomes,[41,51,347] which are then transferred to the 5′ end through long-distance base-pairing between 3′CITE-associated sequences and unstructured sequences within ~100 nt of the 5′ end, followed by ribosome scanning in from or near the 5′ terminus[267] (Fig. 18.6B). Transfer of the 40S subunit from the BYDV BTE to the 5′ end requires both the long-distance RNA:RNA interaction and eIF3, which bridges the BTE and 5′ UTR sequences and increases 40S recruitment by 25-fold.[18] While it was generally accepted that long-distance interactions circularizing viral templates would enhance the translation initiation rate leading to an increase in the number of ribosomes occupying the template, disruption of the long-distance interaction in umbravirus pea enation mosaic virus 2 (PEMV2; family *Tombusviridae*) had no effect on ribosome occupancy but significantly reduced the overall number of translated templates.[51] The presence of key translation elements near the 3′ end of the viral genome ensures that both viral and subviral RNAs have access to the elements, and the close proximity or overlapping of translation elements with structures required for RdRp-mediated transcription from the 3′ end of the genome allows for conformational rearrangements that remove translation factors that could interfere with replication.[155,338]

(+)RNA Virus Replication

Significant advances have recently been made in understanding the replication of plant (+)RNA viruses. As with animal RNA viruses, plant (+)RNA viruses replicate in the cytoplasm and encode only a tiny fraction of the components needed for replication. An expansive number of host factors have been already identified that are co-opted by viruses to reorganize cellular membranes, hijack transport systems, and rewire energy generating pathways to create a cellular environment conducive to rapid and efficient synthesis of progeny while simultaneously avoiding antiviral defenses.[103,209,212] As described in reference[209] the co-opting of host factors to assist in virus replication has led the host to respond with similar proteins to suppress progeny generation, creating an “arms race” that might be exploitable for developing antiviral reagents to protect vulnerable plants. Additional details on plant virus replication can be found in earlier sections on geminivirus, potyvirus, and (−)RNA virus infection cycles.

Most plant and animal (+)RNA viruses construct large, nucleus-sized, single or double membrane-bound protective microenvironments known as virus replication organelles (VRO) that contain the viral replication complexes (VRCs), which for many viruses consist of spherules (single membrane vesicles) and cylindrical channels where replication of individual viral genomes take place.[219,223] Viruses can induce the rearrangement of virtually every type of membrane for VRO and VRC formation, with the virus-specific selection necessitating a massive proliferation and modification of membrane lipids that requires the substantial rewiring of cellular lipid metabolism. Plant virus VROs are located proximal to PD for coupling replication with progeny intercellular movement, and also distal to PD, where progeny are encapsidated for future vector acquisition.[94,157] Viruses establish these membrane environments, which include modification of both phospholipids and sterols,[7,103] by hijacking membrane contact sites (MCS), which are locations where membranes of two different organelles come into close (tethered) apposition. MCS are naturally involved in the nonvesicular, bidirectional shuttling of lipids, Ca^{2+}, amino acids and metals,[254] and a substantial number of ER/PM MCS are located at the entrance of PDs. These MCS are closely associated with VRCs, and SYTA/1, a synaptotagmin involved in generating MCS, is also necessary for formation of normal turnip vein clearing tobamovirus VRCs, with SYTA/1 localizing inside the structures. SYTA/1 binds to MP of many different plant viruses, which likely is key for the spatial coupling of replication and viral progeny movement through associated PDs.[157,169] These recent findings may explain why specific binding of MP to their cognate viral genomes is not required for MP to selectively transport vRNP through PD into adjacent cells.[157,282]

Deciphering the surprising complexity of virus replication has benefited enormously from the ability to replicate components of model (+)RNA viruses BMV (family *Bromoviridae*) (tripartite) and tomato bushy stunt virus (TBSV; family *Tombusviridae*) (monopartite) in yeast to take advantage of the extensive availability of genetic and proteomic resources.[208,213] TBSV encodes five ORFs, two of which are replication-required proteins: a smaller, highly expressed protein encoded by the 5′ proximal ORF (p33) and a ribosome readthrough extension product (p92), which is the RdRp. Genomic and proteomic-based screening of yeast programmed with p33, p92, and a well-studied TBSV defective interfering RNA, revealed that around 500 yeast gene products either support or suppress TBSV replication. In addition to a structural role lining the interior of the VRC peroxisomal membrane spherules, p33 plays an expansive role in assembling VRCs by interacting with about 100 host proteins that participate in VRO and VRC formation and re-direction of host resources[59,209-211] (Fig. 18.7). Some highlights of recent work on TBSV (that is likely applicable to many (+)RNA viruses) include p33 and p92 replication proteins binding to sterols through cholesterol recognition/interaction amino acid consensus (CRAC) and CARC-like binding motifs that are located within the two transmembrane domains of p33.[327] Binding of the two viral proteins to sterols and lipid transfer proteins is thought to aid in the transfer of sterols from ER membranes to the assembling VRO through MCS. p33 also was recently found to retarget small GTPase Rab7 and its cofactors to VRO that results in the co-opting of endosomal vesicles containing proviral components with lipid modifying properties to the sites of replication.[59]

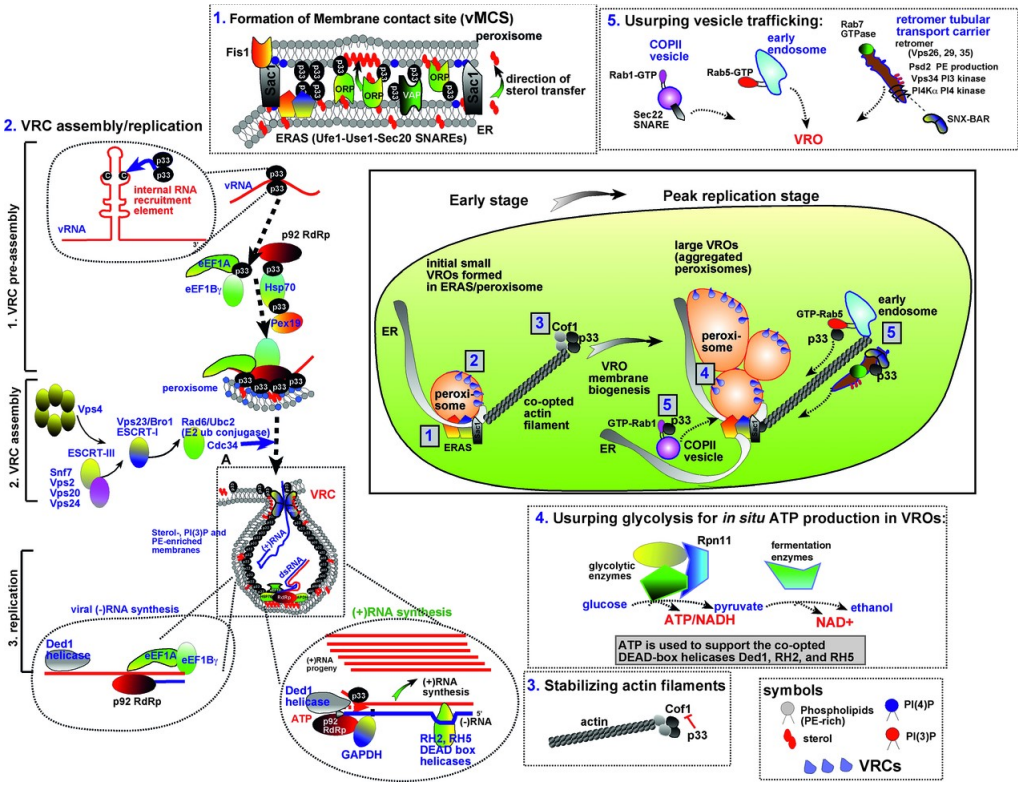

FIGURE 18.7 **Rewiring host pathways and co-opting host factors and membranes during TBSV replication**. Main scheme in the center: TBSV replication is divided into early and peak stages. Note that all the processes described below occur in both stages of replication, but more robustly and with higher intensity in the peak stage. TBSV gRNA is translated resulting in two replication proteins, the more abundant p33, which is the master regulator (*black circle*) of VRO biogenesis, and the p92 RdRp. Process no. 1: Virus-induced formation of viral (v)MCS. The ERAS (ER arrival site of COPI vesicles) subdomain of the ER containing ER SNARE proteins, the Sac1 PI4P lipid phosphatase, and VAP scaffold proteins are targeted by p33 to initiate the biogenesis of VROs. Peroxisomes containing Fis1 mitochondrial/peroxisomal fission protein are also co-opted by p33. By hijacking the cytosolic oxysterol binding proteins (ORPs in plants, called ORBP in mammals) through interaction with p33, vMCSs are formed (as shown in box no.1 on *top*). vMCS serve to enrich sterols, phospholipids, and phosphoinositides within the ER-tethered peroxisomal boundary membranes to optimize lipid composition for TBSV replication. Process no. 2: VRC assembly. During the VRC preassembly process, p33 selectively binds to the TBSV (+)RNA by recognizing a unique internal RNA recruitment element. The p33-viral RNA complex also recruits eukaryotic translation elongation factor 1A (eEF1A) and eEF1Bγ and the p92 RdRp, Hsp70, DDX3-like Ded1/RH20 helicase and with the help of Pex19 chaperone, the complexes are targeted to peroxisomes. During VRC assembly, mono/biubiquitinated p33 recruits the endosomal ESCRTI/III factors and Vps4 AAA ATPase to bend the membranes inward with the VRC preassembly complex inside. This results in a vesiclelike structure with a narrow opening toward the cytosol (as shown schematically). Viral RNA replication starts after Hsp70-driven activation of the p92 RdRp and with the assistance of the RNA chaperone activities of co-opted eEF1A and eEF1Bγ. The synthesis of TBSV (−)RNA leads to double-stranded dsRNA replication intermediates. Then, the dsRNA is partially opened at the 3′ end by co-opted DEAD-box helicases, followed by loading of the p92 RdRp, and (+)-strand RNA synthesis proceeds by strand displacement. The newly synthesized TBSV (+)RNA and sgRNAs are exported out of the spherules to engage in new rounds of translation. Process no. 3: Enhanced recruitment into VROs via stabilization of actin filaments. p33 sequesters Cof1/Adf1 actin depolymerization factor, which leads to stabilization of actin filaments. The actin filaments are then used to deliver co-opted host factors and vesicles into the growing VROs. Process no. 4. Usurping glycolytic and fermentation enzymes for *in situ* ATP production within VROs. Several co-opted enzymes, such as DEAD-box helicases, Hsp70 and AAA ATPases, require plentiful ATP for their functions within VROs. To supply ATP, p33 hijacks the cytosolic glycolytic and fermentation enzymes with the assistance of the proteasomal Rpn11 central interaction hub protein and stabilized actin filaments, which transport into VROs. Process no. 5: Rewiring vesicle trafficking pathways. At the peak stage of replication, the continuously growing VROs require new membrane surfaces to produce many new VRCs/spherules. This is partly accomplished via p33-driven rewiring of the host endomembrane system. The hijacked Rab1-positive COPII vesicles, Rab5-positive early endosomes and the retromer tubular transport carriers all contribute to extra membrane proliferation and delivery of host factors into VROs. Host factors, such as the membrane curvature-sensing endosomal sorting Nexin-BAR proteins and PI(3)P, are proposed to facilitate VRCs/spherules formation. Other co-opted host factors contribute to phospholipid and phosphoinositide production to optimize lipid composition in VROs, which are needed to provide protection of the viral RNAs from cellular nucleases. Altogether, the VROs organize spatiotemporally the replication process leading to the production of hundred thousands of TBSV progeny (+)RNAs per infected plant cells. In the *bottom left box* are symbols of host lipid components subverted by tombusviruses for VRO biogenesis. Note that only a select number of known proviral host factors are presented to simplify the figure. (Figure and figure legend courtesy of Peter Nagy.)

p33 and p92 also bind to the glycolytic ATP generating enzymes phosphoglycerate kinase (PGK1) and pyruvate kinase (PK), redirecting cellular ATP production to the interior of the VROs to support the energy heavy requirements of RNA virus replication.[211] These requirements include activation of chaperone heat-shock protein (HSP)70 by PGK1, which is required along with cochaperones for recruitment and stabilization of p33 and p92 in the VRC membranes, and formation and activation of the replicase complex. PK is necessary for the proviral activity of RNA chaperone DEAD-box helicases, which become permanent residents of VRCs to unwind the 3′ end of the (−)strand portion of dsRNA replication intermediates to allow access to the p92 RdRp for (+)-strand synthesis. Since glycolysis is dependent on regeneration of NAD^+, TBSV also induces expression of fermentation enzymes that reduce pyruvate to ethanol and oxidize NADH. TBSV additionally co-opts translation elongation factors, the actin and ubiquitin networks and the ESCRT machinery for replication, and many more proteins identified in yeast

screens remain to be analyzed. Additional details on TBSV replication can be found in Figure 18.7 and the accompanying figure legend.

The co-opting of host cellular components such as the machinery that generates reactive oxygen species (ROS) in response to various biotic and abiotic stresses[134] into a proviral component for a number of virus-required processes including replication is becoming a familiar scenario in plant-virus interactions.[103,105,209] The p27 replication protein of red clover necrotic mosaic virus (RCNMV; family *Tombusviridae*) associates with the host ROS-generating machinery, relocating the NADPH oxidase enzyme and its regulators from the PM to the perinuclear ER and triggering rapid intracellular ROS bursts that are required for robust viral RNA replication.[104] BMV, which similarly requires ROS bursts for replication, encodes replication protein 1a that functions as a viroporin, generating pores in the ER membrane that release oxidizing power from the ER lumen. This is proposed to form di-sulfide bonds linking 1a proteins together to form a crown on the neck of replication spherules, which is necessary for late functions in replication.[104,223]

Plant Virus Movement

Plant viruses establish replicative infection in leaf epidermal, mesophyll, phloem, or root cells, following introduction by a vector or through mechanical damage. Viral genomes spread between neighboring epidermal and mesophyll cells through PD that connect the cytoplasm between cells. PD nanochannels are lined by the cell wall and PM of adjoining cells. The central channel, known as the desmotubule, is derived from the ER stretching through the PD. This symplastic continuity has been referred to as the "supracellular nature" of plant cells and enables passive diffusion of low molecular weight signals, nucleic acids, and proteins between neighboring cells.[175] Transfer of larger proteins (>60 kDa) across PD occurs via the noncell-autonomous pathway (NCAPP) whereby the large protein or its chaperoning partner actively expands the size exclusion limit (SEL) of the PD, dilating the central channel to enable cell-to-cell transfer.[156] PD proteomic studies have identified more than 1300 PD-associated proteins, some of which are likely involved in movement of endogenous RNAs or viruses between cells.[61] Calcium signaling and callose deposition are highly regulated at PD by proteins such as Ca^{2+} calmodulinlike binding protein (CML41), calreticulin, and β-1,3-glucanase, which modulate callose deposition at the cell wall. Noncell autonomous MP-like cellular proteins have also been identified, including cucumber PHLOEM PROTEIN 2 (PP2) and PHLOEM PROTEIN 16 (PP16), maize KNOTTED1(KN1) transcription factor, and Arabidopsis FT.[21,33,127,175,188,240]

Linkage Between Virus Replication and Intracellular Movement to the PD

The first virus MP characterized was the 30K MP of tobacco mosaic virus (TMV; family *Virgaviridae*).[45] 30K binds nonspecifically to viral RNAs like beads on a string and is responsible for increasing the PD SEL and transporting linear nucleic acids into neighboring cells. The only identified PD localization signal (PLS) on viral MPs or RNAs is in the N-terminal 50 amino acids of the TMV MP, which contains two amino acids (Val-4 and Phe-14) critical for PLS function.[337] The TMV PLS sequence interacts with Arabidopsis SYTA/1, which promotes its association with PD. The Arabidopsis SYTA/1 is a Ca^{2+} sensor that regulates endocytosis. As described in the section on replication, SYTA/1 also associates with ER-PM MCS and is recruited by MPs encoded by TMV, turnip vein clearing virus (TVCV; family *Virgaviridae*), cabbage leaf curl virus (CaLCuV; family *Geminiviridae*), and TuMV (family *Potyviridae*).[157,158,287]

TMV is emblematic of a wide range of viruses whose replicating genomes are found within VRCs. These factories also include actin and microtubules that affect the mobility of VRCs and drive interactions with other viral proteins.[94,221] Microtubules act as a scaffold for anchoring VRCs as they grow and mature into X-bodies or inclusions. The ER is associated with actin filaments that along with myosin motors enable lateral movement of proteins. The TMV MP, which binds to a microtubule-associated protein that plays a regulatory role in virus cell-to-cell movement,[249] accumulates in the vicinity of VRCs and directs some to the vicinity of PD where either newly synthesized viral genomes or intact VRCs migrate across PD into neighboring cells[50,221] (Fig. 18.8).

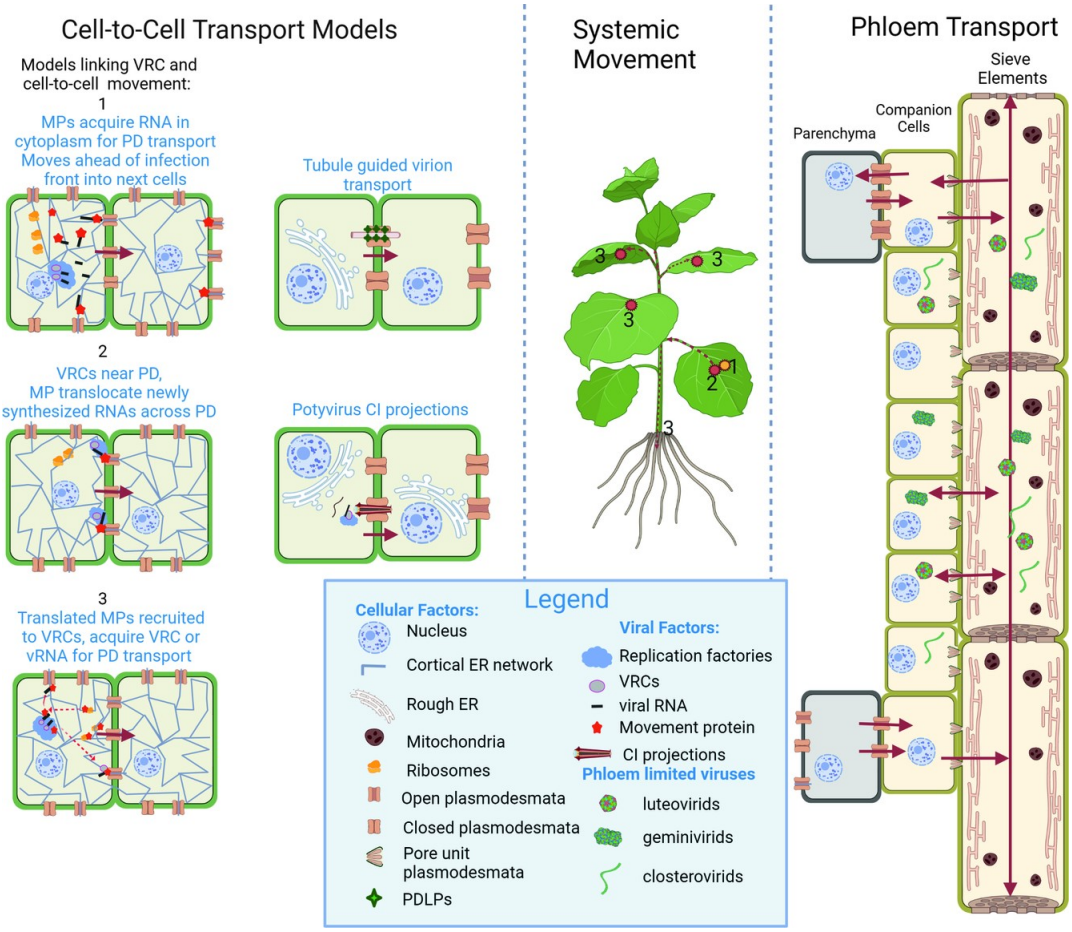

FIGURE 18.8 **Virus movement mechanisms from the cellular to whole plant levels**. The legend identifies cellular and viral factors in each diagram. For viruses that closely integrate VRCs and PD transport, there are three models that depict how viral MPs engage with newly synthesized genomes or VRCs to drive infection into neighboring cells. Two other models are for viruses that produce tubules and for potyviruses that require CI projections. Regarding systemic movement, infection starting in the *orange circle* (1) is primary movement from cell-to-cell in the inoculated leaf followed by (2) entry into the vasculature for movement to the upper leaves. Step (3) is unloading in upper leaves. *Arrows* indicate direction of movement in the plant. Regarding phloem transport, there are three genera of viruses that are examples of phloem limited viruses that are restricted to infection of the phloem parenchyma, companion cells, and sieve elements (listed at *top* of diagram). *Red arrows* show source to sink direction of movement as well as the exchange between neighboring cell types.

Other taxonomic groups of plant viruses encode several MPs that act together to move viral genomes from cell-to-cell. Some viruses encode small membrane binding proteins in conserved gene modules known as the binary movement block (BMB) and triple gene block (TGB), which typically include an RNA binding protein and one or more membrane proteins.[201] A representative BMB virus is melon necrotic spot virus (MNSV; family *Tombusviridae*) whose membrane-associated MPs traffic viral RNA via the Golgi apparatus to PD.[75,153,215] The TGB of potato virus X (PVX; family *Alphaflexiviridae*) has an N-terminal RNA binding domain and C-terminal RNA helicase domain. TGB1 functions as both the viral silencing suppressor and the cell-to-cell MP and has the capacity to destabilize virions to promote translation of viral RNAs.[205,280] BMB2 as well as TGB2 and TGB3 are membrane-binding proteins that associate with the ER, ER-derived granular vesicles, and ER-derived bodies near PD.[120,147,153,280] TGB2 has RNA-binding activity, and the vesicles may scaffold ER-anchored replication complexes for transport toward PD. The viral CP is also essential for cell-to-cell movement. The PVX X-body is an organized inclusion structure of remodeled actin, ER, and Golgi membranes that is arranged around large aggregates of the TGB1 protein. These structures compartmentalize viral gene products, granular vesicles, ribosomes, virions, and VRCs.[14,169,281,297]

Replication and trafficking of alphaflexivirids are closely linked and TGB-associated VRCs remain active in virus replication at the entrance of PD.[157] As described in the section on (+)RNA virus replication, separate viral factories appear to produce VRCs for movement of newly synthesized genomes[94,280] and for packaging and vector acquisition. However, other reports suggest that all active VRCs accumulate near PD, and X-bodies that form at late stages of infection are for removal of aggregated and overexpressed proteins.[94] Interestingly, the PVX TGB1 interacts with remorin (REM), which associates with lipid rafts and locates in microdomains along the PM and PD. Studies show that REM interferes with PVX cell-to-cell movement by binding directly to the TGB1 protein.[231,238] Bamboo mosaic virus (BaMV; family *Alphaflexiviridae*), a close relative of PVX, can be associated with a satellite virus (satBaMV) whose encoded p20 protein interacts with fibrillarin in the nucleolus and with PDs and enables independent satBaMV movement.[31] *N benthamiana* high mobility group (HMG) 1/2a protein, a nonhistone protein associated with chromatin, Rap-GAP1, and thioredoxin h protein, serves as a positive and negative regulator of BaMV cell-to-cell and systemic movement.[4,34,101] The protein kinase CK2 also regulates virus cell-to-cell movement through phosphorylation of the CP in a manner that regulates RNA binding.[102]

The dynamic cellular interactions involving TGB proteins of the multipartite *Virgiaviridae* differ from the monopartite viruses (ie, PVX, BaMV). For these viruses, TGB1 does not increase PD SEL, lacks silencing suppressor activity, and accumulates in the nucleolus in association with

fibrillin or coilin.[176,255,256,314] Potato mop-top virus (PMTV) TGB1 shuttles into the nucleus mediated by importin-α, and defects in nuclear import restrict virus systemic movement. For many of these viruses, fibrillarin is transported out of the nucleus and joins the ribonucleoprotein complex that is trafficked long distances. TGB1 complexes with TGB2 or TGB3 in the cytoplasm for cell-to-cell movement.

As described earlier in this chapter, potyvirus movement is via replication vesicles that appear to be transported to healthy surrounding cells via PD.[80] In addition to P3N-PIPO and CI, viral CP is essential for TuMV movement.[40] Both CP and CI are abundantly present in the PD proteome of TuMV-infected plants[227] and the specific nature of the molecular complex that moves viral genomes between adjacent cells is not entirely understood. Current data suggest that replication vesicles move between mesophyll cells and enter into the vasculature, but PD transport also requires the viral CP and possibly viruslike structures. Furthermore, HC-Pro and AGO1 of PVA and TuMV are required for virion stability.[232] While partially formed virions can move cell-to-cell, they weakly support long-distance movement and mature virions are essential for phloem transport. In addition, TuMV replication vesicles and virions are visible by microscopy in xylem tissues.[299] These seemingly contradictory data suggest the possibility that VRCs as well as virions have essential roles in the spread of infection.

The 70 kDa cylindrical inclusion protein (CI) contains two helicase domains that are required for replication and movement. P3N-PIPO, generated from ribosome recoding, recruits CI, cellular Ca^{2+} binding protein PCaP, and α-expansin EXPA1 creating a complex associating with PD. CI oligomers form conical projections that aggregate near PD and either act as a funnel or beacon for cell-to-cell movement.[38,195,202,203,311] VPg and CP participate in cell-to-cell movement either by acting to remodel PDLPs or by directing virions toward PDs.[301] Alternative models suggest that either virions or viral (v)RNA/CP complexes are guided to the CI conical structures for transport to neighboring cells, or that VRCs themselves are transported across PD, since the 6K2 protein induces motile ER-derived vesicles containing vRNA.[80,301]

Other RNA and DNA icosahedral viruses such as cowpea mosaic virus (CPMV; family *Secoviridae*), grapevine fanleaf virus (GFLV; family *Secoviridae*), tomato spotted wilt virus (TSWV; family *Tospoviridae*), and cauliflower mosaic virus (CaMV; family *Caulimoviridae*) produce MPs that form tubules, which extend between neighboring cells for transport of mature virions.[8,124,233,271] These hollow tubules displace the desmotubule within the PD lumen and become the conduit for virions that march through the tubule into the neighboring cells.[44] Notably, PDLP isoforms interact with the CPMV, GLFV, and CaMV MP and appear to act as host receptors mediating PD targeting, although their functions in virus movement are not fully established.[8,44] Myosin XI-K and myosin XI-2 motors drive anterograde transport from the ER-to-Golgi-to-PM. The proposed model for GFLV is that the MP reaches PD either by diffusion or microtubule mediated transport. The PDLPs traffic along the secretory system to PD in a manner that is dependent upon myosin XI-K and myosin XI-2.[8] At the PD, the PDLP promotes assembly of the GFLP MPs into tubules, which then conduct virions into neighboring cells.[8]

Geminiviruses encode nuclear shuttle proteins (NSP/CP) and MP to facilitate the transport of nascent viral DNA from the nucleus to the cytoplasm and through PD to adjoining cells.[185] CP acetylation promotes viral (v)DNA-NSP complex formation and vDNA transport to the cytosol, where the infectious genomes move to neighboring cells and then systemically throughout the plant. The exact composition of the movement-competent complex is unclear. The NSP and MP interact together and with the vDNA and host proteins.[185] A complex composed of histone H3, NSP, MP, and vDNA was identified from bean dwarf mosaic virus (BDMV; family *Geminiviridae*)-infected leaves. H3 colocalizes with NSP in the nucleolus and MP at punctate bodies at the cell wall. The interaction between NSP and MP may mediate the export of the H3-associated movement complex from the nucleus via nuclear pores to the PD. NIG, an NSP-interacting GTPase that shares transport properties with the human Rev-Interacting Protein may be involved in release of the NSP-vDNA complex from the nuclear periphery to the cytoplasm. NSP-interacting proteins that display proviral functions and suppress host antiviral defenses are reviewed in Ref.[185]

Long-Distance Movement Into and Out of the Phloem

Viruses move in the direction of the vasculature, entering bundle sheath cells and then into the vascular bundles that include xylem, phloem, and parenchyma. The bundle sheath is a primary barrier for virus entry and exit to the vasculature and requires compatible interactions between viral MPs and host proteins to transit across this barrier. Once past bundle sheath cells, viruses enter phloem companion and vascular parenchyma cells. Specialized PD pore units (PPUs) provide another selective barrier for virus transfer into phloem sieve elements.[65] Sieve elements are enucleated cells that stack end to end, comprising the phloem that conducts a wide range of molecules throughout the plant. Virus movement through the phloem is passive, with transport following along with the upward or downward flow of photoassimilates and other molecules. Unlike cellular proteins and smaller molecules, viruses do not move freely across the PPU but require the activities of MPs. Many (+)RNA viruses whose genomes contain 3′ tRNA-like structures require these elements for phloem loading and transport.[159]

As described above, many viruses rely on two modes of long-distance transport, as virions or vRNPs, and the choices between these modes may be host selective. Other viruses may exclusively require viral particles only for long-distance movement.[98] For cucumoviruses, potyviruses, and tombusviruses, the surface-exposed domains of the CP allow direct interactions with host factors that facilitate phloem mobility. Viral MPs with their capacity to modify PD SEL can also be required for entry into and out of the phloem. Potyviruses require VPg linked to the 5′ end of the viral RNA, as well as the CP and 6K2 membrane binding protein.[300]

A few cellular proteins that aid viral transport in the phloem have been identified. For example, umbraviruses do not encode a CP and thus cannot produce virions. Instead, the umbravirus ORF3 protein enters the nucleus and relocalizes nuclear fibrillarin to the cytoplasm to integrate into vRNP complexes,[132] which then move systemically. Fibrillarin is also adopted by other viruses and subviral entities for long-distance movement, including rice stripe tenuivirus, potato mop top pomovirus, and satRNA of BaMV (family *alphaflexiviridae*).[65,98,121]

Species belonging to *Closteroviridae* and the family previously known as the *Luteoviridae* are delivered by phloem-feeding hemipteran insects and are limited to infecting phloem parenchyma, companion cells, and sieve elements.[270] Closteroviruses form long flexuous filamentous virions and their movement is associated with virion assembly. Particles have unique tail structures composed of the viral MP Hsp70h proteins and a p20 protein at the tip of the tail, which docks with host receptors at PD. Lettuce infectious yellow virus is a member of the genus Crinivirus, within the *Closteroviridae*, and also produces conical structures at PD that are functionally reminiscent of the CI structures produced by potyviruses.[237] In addition, the leader protein (L-Pro) is necessary for long-distance transport but is not associated with virions.[98]

Members of family *Reoviridae* that infect plants include genera Fijivirus, Oryzavirus, and Phytoreovirus, and many (but not all) are phloem

limited. These viruses infect primarily monocot plants and have nonmembrane-bound icosahedral virions. Their genomes are multipartite, (ds)RNAs with up to twelve genome segments and induce galls, tumors, or vein swellings. Reoviridae viruses create cellular hyperplasia in the phloem and modify the arrangement of phloem sieve element-companion cell complexes in the vasculature of maize and rice leaves and stems. Within tumors, these viruses create unique structures known as “flexible gateways” at the interface between sieve elements.[178,258,326]

Viruses have also been detected in the xylem, the nonliving cellular tubes that transport water. For example, wheat soilborne mosaic virus (WSBMV; family *Virgaviridae*) is transmitted by a plasmodiophorid vector into growing root cells and establishes infection in immature xylem parenchyma. As roots grow and xylem matures, virions and virus inclusion bodies are seen in xylem vessels and guttation fluid.[294] Other viruses detected in the xylem include icosahedral and filamentous viruses such as rice yellow mottle virus (RYMV family Solemoviridae), TBSV (family *Tombusviridae*), cucumber green mottle mosaic virus (CGMMV family *Virgaviridae*), PVX (family *Alphaflexiviridae*), zucchini yellow mosaic virus (ZYMV; family *Potyviridae*), BMV (family *Bromoviridae*), TMV (family *Virgaviridae*), pepper mild mottle virus (PMMV; family *Virgaviridae*), citrus tristeza virus (CTV; family *Closteroviridae*), and TuMV (family *Potyviridae*).[17,48,182,200,226,273,300]

Viroids are small single-stranded circular RNAs that replicate in host cells but do not encode proteins. They are the smallest noncoding RNA infectious agent in plants ranging in size from 250 to 400 nt. While a few examples such as potato spindle tuber viroid (PSTVd; family *Pospiviroidae*) spread systemically throughout the plant, many viroids are phloem limited. All viroids use components of the host for replication and transport. Investigations of PSTVd identified many unique three-dimensional loop structures and 12 loops were identified as involved in systemic trafficking. In particular, Loop19 is flanked by *cis*-Watson-Crick base pairs, categorized as an A/C loop and mutations impede cell-to-cell movement but not replication.[114] Distinctive motifs control trafficking from the epidermis to the mesophyll and parenchyma, across bundle sheath cells into companion cells, and then into sieve elements.[303] The tomato Virp1 was shown to bind to the TR hairpin of PSTVd and is associated with phloem transport.

PP2 is an RNA-binding protein with viral MP properties that is associated with phloem transport of HSVd (family *Pospiviroidae*).[292] Surprisingly, PP2 is also implicated as a MP for ulaRNA CYVaV-citrus (Simon, unpublished). PP2 binds specifically to CYVaV, conferring partial protection from RNases and allowing the replicon to transit through the vascular system of a large number of unrelated plants including citrus, cucumber, tomato, papaya, and grapevine. Specific binding to PP2 correlates with the appearance of virion-sized “bundles” that aggregate both *in vitro* and in phloem sap.

Host Responses in Defense and Immunity

The major forms of plant antiviral immune responses are (1) RNA silencing (RNA interference [RNAi]) and other associated pathways; (2) resistance (*R*) gene-mediated responses triggered by intracellular recognition of pathogen effectors (effector-triggered immunity [ETI]); (3) pattern-triggered immunity (PTI) based on recognition of pathogen-associated molecular patterns (PAMPs), which lead to a nonspecific systemic response (systemic acquired resistance or SAR); and (4) the unfolded protein response (UPR).

RNA Silencing and Associated Pathways

Vector-assisted entry of plant viruses injures the plant, which rapidly triggers Ca^{2+} influxes,[329] a prerequisite for activation of the calmodulin-binding transcription activator CAMTA3. Activated CAMTA3 relieves suppression of the salicylic acid (SA) defense pathway,[133] thus improving biotic and abiotic stress tolerance[107] that is linked to RNAi through direct activation of RNA-dependent RNA polymerase 6 (RDR6) and bifunctional nuclease-2 (BN2).[305] BN2 stabilizes the key RNAi components AGONAUTE1/2 (AGO1/2) and DICER-LIKE1 (DCL1) by degrading the microRNAs (miRNAs) that normally target their transcripts.[305] Virus infection also induces miRNAs that regulate plant’s defense responses, as demonstrated by potyvirus soybean mosaic virus (SMV; family *Potyviridae*) infection of soybean, where upregulated miRNAs control expression of many key silencing components and the NBS-LRR family of resistance genes.[15]

Antiviral RNAi in Plant Virus Infection

Once triggered, antiviral RNA silencing begins with endonucleolytic cleavages of double-stranded regions of the gRNA by DCLs with assistance of dsRNA-binding proteins (DRBs), leading to the biogenesis of 21-24 bp viral small interfering RNA (vsiRNA) that are stabilized by 3′ end methylation catalyzed by Hua Enhancer 1 (HEN1). The guide strand of the vsiRNA duplex that is complementary to the gRNA target assembles with AGO proteins to form the RNA-induced silencing complex (RISC) or RNA-induced transcriptional silencing complex (RITS). RISC, which targets RNA genomes and transcripts of DNA viruses for posttranscriptional gene silencing (PTGS), limits virus infection via the 21 and 22 bp vsiRNAs produced by DCL4 and DCL2. Silencing of target viral RNAs occurs by the slicing activity of the RNase H endonuclease fold within AGO or by RISC-mediated translational repression. Although most AGO proteins target nonprotected viral RNAs, only a subset (AGOs 1/2/3/4 /5/7/10 in Arabidopsis and AGOs 1/18 in rice) appear active in antiviral defense.[264]

While defense by AGO-mediated slicing is well studied, less is known about RISC-mediated viral RNA translational repression.[56] Translational repression requires the association of ribosomes with sRNAs, their viral RNA targets, and AGO1/AGO2. AGO1 was shown to repress translation of nepovirus RNA2[77] and translation of a reporter mRNA carrying the 5′ UTR of a potyvirus was inhibited by a vsiRNA complementary to the UTR.[110] Translation repression was thought to occur by steric hindrance of ribosome movement by RISC binding either at the initiation or elongation phase. AGO1 associates with polysomes during potyvirus infection,[109] but it remains to be shown if it has a role in translational repression.

Replication in membranous compartments protects viral RNAs from RNA silencing, while progeny extruded into the cytoplasm must be protected by virus-encoded suppressors of RNA silencing (VSRs). Maintaining silencing suppression and viral gene expression during PVA (family *Potyviridae*) infection requires assembly of a high molecular weight protein complex consisting of the potyvirus VSR (the HC-Pro protein), host protein VARICOSE, and other viral and host proteins[43] (Fig. 18.5). AGO 1 is co-opted by PVA to play a positive role during potyvirus infection by interacting with HC-Pro within a large protein complex to stabilize PVA particles, providing an additional example of the evolutionary arms race between viruses and their hosts.[232] This leaves AGO2 as the main antiviral effector in potyvirus infection.[70]

Antiviral defense against DNA geminivirus genomes occurs by transcriptional gene silencing (TGS)[87] when viral dsRNAs formed by highly

structured or overlapping bidirectional transcripts are cleaved by DCL3 into 24 nt siRNAs and loaded into AGO4 to form RITS. RITS includes the RNA-directed DNA methylation apparatus to methylate cytosines in targeted viral DNA sequences to suppress viral minichromosomes. Geminivirus infection also induces the biogenesis of repeat-associated (ra)siRNAs from Pol VI or Pol V transcripts that associate with AGO4 and mediate plant DNA methylation, which contribute to host defense along with histone post-transcriptional modifications, chromatin remodeling, and nucleosome repositioning. As a counter defense strategy, geminivirus proteins interact with components of the methylation pathway to help the virus escape host defense (Fig. 18.4).[339]

Antiviral defense against (ds)RNA and (−)RNA viruses is not identical in monocotyledonous and dicotyledonous plants: for example, R-genes such as NLRs do not appear to play a major role in rice antiviral defense.[348] Phytohormone jasmonic acid (JA) accumulates in response to viral infection and induces AGO18 transcription through the previously established JAMYB transcriptional regulation pathway.[332,333] AGO18 sequesters the two miRNAs, miR168 and miR528, which normally negatively regulate AGO1 and l-ascorbate oxidase (AO).[317,318] AGO18 acts as a decoy, whereby miR168 and miR528 are loaded into AGO18, thus preventing binding to their targets, AGO1 and AO. AGO1 binds vsiRNAs to cleave viral gRNAs through RISC, while AO elevates the base ROS levels to restrict viral infection.

Secondary sRNA Production in Antiviral RNAi

Host RDRs together with SUPPRESSOR OF GENE SILENCING 3 (SGS3) use primary vsiRNAs as primers for conversion of viral RNA into dsRNA for biogenesis of secondary vsiRNA. This RNAi amplification step allows for production of trans-active sRNAs (tasiRNAs) that target viral RNA sequences external to the original RISC target site, a phenomenon known as transitive silencing. Transitive silencing allows for cell-to-cell and systemic spread of silencing to distal tissues via the phloem. Noncell-autonomous mobile silencing requires a DCL2-dependent genetic network and 22 nt siRNAs as mobile signals,[345] which play a role in tissue recovery during viral infection.[145] Plasma membrane-localized receptorlike kinases BAM1 and BAM2 play redundant roles in cell-to-cell spread of the mobile silencing signal[248] and are targeted by the geminivirus C4 protein and tombusvirus p19,[79,248] suggesting that both VSRs may halt cell-to-cell spread of RNA silencing.

Using an *in vitro* system for generating secondary siRNAs, RDR6 recruitment to viral RNA templates required SGS3 (a putative nuclear RNA export factor), SILENCING DEFECTIVE5, primary siRNAs, and AGO.[252] Essential components for biogenesis of secondary vsiRNAs also includes AMINOPHOSPHOLIPID TRANSPORTING ATPase 2 (ALA2), the related ALA1,[85] and ANTIVIRAL RNAI-DEFECTIVE2 (AVI2).[86] ALA2/1 and AVI2 are also important for production of host siRNAs induced during virus infection (vasiRNAs), which guide AGO2-mediated RNAi of hundreds of host genes correlating with broad-spectrum antiviral activity.[25] ALA1 and ALA2 are cellular flippases, which aid cross-membrane movement of phospholipids between the two leaflets of the PM, suggesting that biogenesis of secondary vsiRNA and vasiRNA molecules may require a membranous environment.

Interactions of VSRs With Host Pathways

Plant viruses encode VSRs to suppress the host's silencing machinery, interfering with sRNA biogenesis, RISC assembly/function, and systemic silencing.[332] VSRs are multifunctional, also interacting with other cellular pathways involved in immune responses. For example, cucumber mosaic virus (CMV; family *Bromoviridae*) 2b, potyviral P1/HC-Pro, geminiviral C2, and satellite βC1 inhibit antiviral signaling mediated by phytohormones SA and JA.[28,349] SA normally leads to suppression of RDR1-mediated regulation of basal virus resistance, restriction of meristem invasion by viruses, and symptom amelioration and recovery. In addition, SA is involved in mitochondrial ROS accumulation and redox signaling and their regulation by alternative oxidase and glutathione, a pathway that serves to limit virus accumulation and spread and aids in host plant recovery.[206] SA-mediated induction of autophagy during CMV infection promotes VSR 2b degradation and RNA silencing and negatively impacts SA-immunity driven disease development.[262] This complex interplay between SA, 2b, RNA silencing, and autophagy is thought to balance virus accumulation and plant fitness using these two parallel pathways.

The multifaceted interactions of geminiviral VSR induce activation of endogenous VSRs that suppress PTGS, downregulate a histone methyltransferase to suppress TGS and direct inhibition of host methionine cycle enzymes S-adenosyl homocysteine hydrolase (SAHH), adenosine kinase (ADK), and S-adenosylmethionine decarboxylase (SAMDC) to suppress both PTGS and TGS[87,291] (Fig. 18.4). Due to inactivation of ADK, cellular cytokinin levels increase, which affect cell cycle regulation and positively impact viral replication.[12] Cellular energy metabolism and stress-related SUCROSE NONFERMENTING-related protein kinase (SnRK1) are activated upon geminivirus infection,[260] with SnRK1 phosphorylating βC1, which negatively affects its RNA silencing suppression activity and results in reduced infection[351] (Fig. 18.4). The βC1 satellite also suppresses the antiviral activity of the mitogen-activated protein MAPK pathway that is activated during geminivirus infection[100] (Fig. 18.4).

Like geminiviral VSRs, the activities of potyviral HC-Pro in silencing suppression and other antiviral responses are versatile[289] (Fig. 18.5). HC-Pro is responsible for formation of RNA silencing suppression-related granules that contain gRNA.[88] These proviral RNA granules are subjected to NEIGHBOR OF BRCA1 (NBR1)-dependent selective autophagy, suggesting autophagy is a second layer of antiviral response after RNAi.[90]

Surveillance Pathways of Cellular mRNA Metabolism and Plant Virus Infection

RNAi and RNAi suppression networks interact with the cytoplasmic RNA regulatory network of the infected host cell.[181] Interaction between PTGS and the 5′-3′ decay pathway of mRNAs during potyvirus infection suggests that RNA fragments partially cleaved by exonuclease XRN4 following mRNA decapping may serve as templates for RDR6 in dsRNA synthesis.[160] In potyvirus infection, HC-Pro interacts directly with and inhibits XRN4 activity, whereas VPg interacts with decapping factor DCP2 and sequesters it to nucleus reducing mRNA decay.[160] In addition, VPg directs RDR6 and SGS3 to ubiquitination-dependent autophagy degradation[36] (Fig. 18.5).

NMD, which detects and eliminates mRNAs with premature termination codons (PTC) or mRNAs with naturally long 3′ UTRs (over 200 nt in plants), is another cellular mRNA surveillance pathway that targets viral RNAs encoding multiple ORFs whose stop codons are recognized as PTCs.[129] Viruses have evolved mechanisms to escape decay through the action of *cis*- or *trans*-acting viral factors.[191] For example, p26, the long-distance movement protein of PEMV2 (family *Tombusviridae*) binds to viral and cellular mRNA 3′ UTRs, restricting the binding of NMD

factors.[190] TCV (family *Tombusviridae*) counters NMD with unstructured sequences just downstream from the CP termination codon.[192] N6-methyladenosine (m6A) abundance, which is a nucleotide modification known to regulate life cycle of animal viruses, appears to co-operate with the NMD in antiviral regulation of alfalfa mosaic virus (AMV; family *Bromoviridae*) gRNA.[184]

Autophagy and Plant Virus Infection

Autophagy is an evolutionarily conserved degradation mechanism that regenerates cells by eliminating unnecessary and/or harmful cell organelles and macromolecules. Autophagy is activated in response to cellular stress conditions and appears to play a central role in antiviral defense.[150] Interactions of plant viruses with autophagic processes are as complex and diverse as those of animal viruses and vary between antiviral and proviral processes.[99,323] Delivery of VSRs and other viral proteins to autophagic degradation limits infection of both plant DNA and RNA viruses. CaMV (family *Caulimoviridae*), a pararetrovirus with a dsDNA genome, accumulates in the cytoplasm, where NBR1 cargo receptor delivers them to autophagy and vacuolar destruction unless they are protected in cytoplasmic inclusion bodies comprised of CaMV P6 protein and particles.[89] VSRs HC-Pro and 2b are subjected to autophagy in association with a calmodulinlike protein (rgs-CaM)[214] and the βC1 suppressor of cotton leaf curl multan virus (CLCuMuV; family *Geminiviridae*) is subjected to degradation via its binding to ATG8.[93]

VSRs also promote infection by escorting REMORIN 1,[67] a protein that inhibits cell-to-cell movement, to autophagy. Interestingly, RSV infection is downregulated by autophagy-mediated degradation of VSR p3 through its interaction with host protein P3PI[116] and by proteasomal degradation via its interaction with ubiquitinlike protein 5.[32] Furthermore, an RSV-derived vsiRNA supports activation of antiviral autophagy by downregulating the mRNA of eIF4A, a negative autophagy regulator.[346]

Ubiquitin Proteasome System, SUMOlylation, and Plant Virus Infection

Ubiquitin is a highly conserved eukaryotic protein that becomes attached to cellular or viral proteins as a post-translational modification that identifies them for degradation by the 26S proteasome. Studies have shown that the ubiquitin proteasome system (UPS) plays a role in plant proviral and antiviral functions by degrading viral replicases, VSRs, MPs, and CPs to limit virus infection.[52] Many plant viruses exploit UPS by targeting E3 ligases or other components of the SCF (SKP1/Cullin1/F-box/RBX1) complex. F-box proteins contribute to SCF complexes, which form a scaffold for recruiting target proteins for ubiquitination. For example, geminivirus beet severe curly top virus (BSCTV)-encoded C4 protein promotes expression of the RING-type E3 ligase RKP, which in turn degrades cell cycle inhibitors (ICK/KRPs).[152] Geminivirus replication is linked to cell cycle regulation and disruption of host cell cycle control is beneficial for virus infection. Turnip yellow mosaic virus (TYMV; family *Tymoviridae*) replicase protein contains a cysteine protease domain that de-ubiquitinates the viral polymerase and prevent its proteasomal degradation.[63] The rice grassy stunt virus (RGSV; family *Nairoviridae*) P3 protein includes a U-box type E3 ubiquitin ligase that targets the nuclear RNA polymerase D1a (NPRD1a). NPRD1a in rice is an RNA polymerase IV enzyme, which is required for RNA-directed DNA methylation and its degradation via UPS enhances host susceptibility to RGSV infection.[342] A tobacco ubiquitin E3 ligase RFP1 interacts with βC1 and mediates its ubiquitination and proteasomal degradation, which leads to symptom attenuation (Fig. 18.4).[259]

SUMOylation involves ligation of a 10 kDa small ubiquitin-related modifier, SUMO, to modulate protein activities, interactions, and subcellular localization. Interaction of SUMO conjugating enzyme SUMO3 with RNA polymerase NIb of TuMV regulates the nuclear-cytoplasmic partitioning of NIb and attenuates the host NONEXPRESSER OF PR GENES1 (NPR1)-mediated antiviral defense, thereby promoting infection.[37] NIb's activity is inhibited via its direct interaction with ATG6/Beclin1, which forms a protein complex that likely acts as the cargo receptor for autophagic degradation of NIb through a specific interaction with ATG8a.[161] SUMOylation of Rep proteins of geminiviruses likely creates a favorable environment for geminivirus replication as well.[10]

Effector-Triggered Immunity and Cross-Talk With Other Cellular Pathways

Effector-triggered immunity (ETI) is activated upon specific recognition between a viral factor and the corresponding nucleotide-binding leucine-rich repeat receptor (NLR) that is encoded by a dominant R (resistance) gene. Plant genomes encode hundreds of R genes for resistance to fungal, bacterial, viral, and other pathogens.[11] Plant NLRs carry either a Toll-Interleukin-1-receptor (TIR) homology domain or coiled-coil (CC) domain at their N-terminus. The main domains of NLRs, the CC or TIR domain, the leucine-rich repeat (LRR) domain, and the nucleotide binding (NB) domain play important roles in recognition and translating the recognition into a defense response. Molecular interactions leading to perception are still poorly understood. Broad-spectrum tospovirus resistance is conferred when the LRR domain of tomato Sw-5b, which is a CC-NLR, directly associates with a conserved 21 amino acid peptide within the NSm MP (NSm^{21}), which activates Sw-5b by releasing the interaction between NB and LRR domains.[354] Activation of TIR-NLR N that confers broad-spectrum tobamovirus resistance upon recognition of the p50 helicase domain depends on N self-association/dimerization and other unidentified factors, since this is not sufficient for downstream signaling.[193]

Recognition often requires more than a simple effector/NLR interaction and can include coreceptor sensor proteins bound to the N-terminal portion of NLRs.[131] The essential role of coreceptors in NLR function is supported by the widespread presence of integrated decoys recognizing pathogen effectors in NLR sequences.[148] The Solanaceae domain (SD) in the N-terminal portion of Sw-5b,[35] whose 3-D structure was recently solved,[164] increases the sensitivity of NSm perception by providing a second binding site for NSm^{21}.[163] A high-resolution cryo-EM structure of a wheel-like pentamer protein complex called ZAR1 resistome revealed a pentamer of the Arabidopsis CC-NLR ZAR1 receptor that functions in recognition of a bacterial pathogen, associated with coreceptors pseudokinase RKS1 and kinase PBL2. These data demonstrate the oligomerization upon dATP binding that is required for immuno activation[302] and will likely provide further insight into the function of NLR receptors that recognize viruses. Figure 18.9 shows more examples of the most studied NLR receptors, the proteins involved in the function of each receptor, and the localization of the function in the cell.

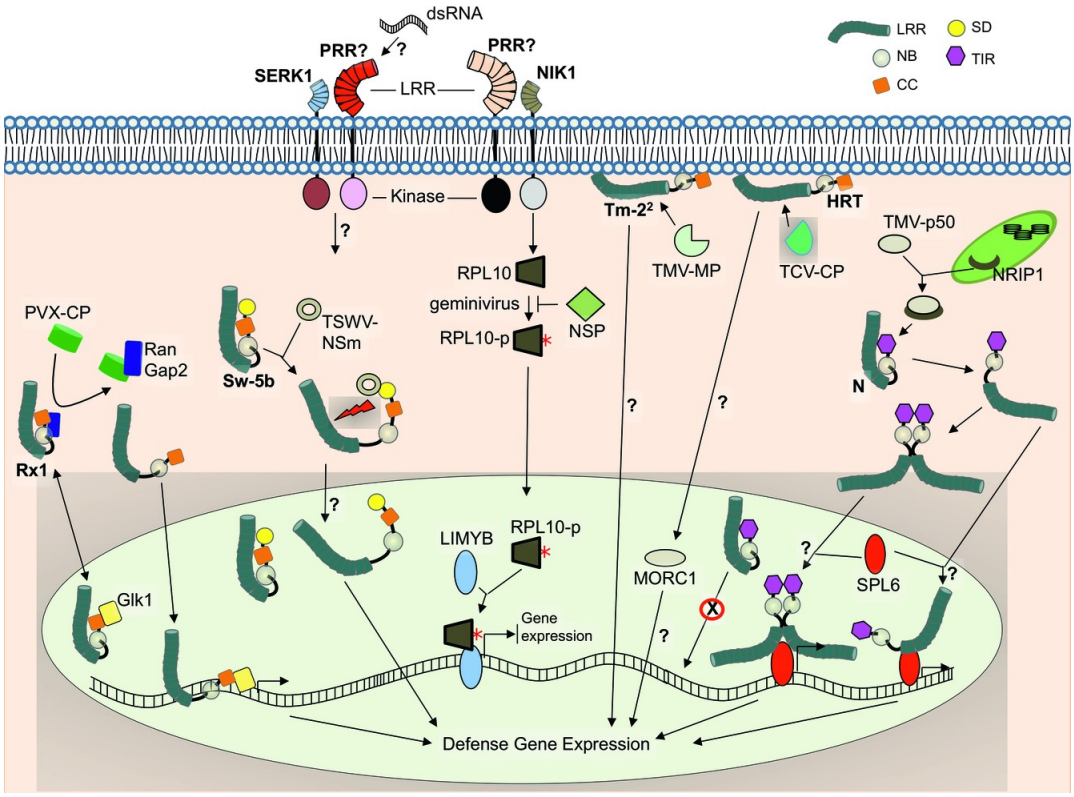

FIGURE 18.9 **Recognition of viral pathogens by cell surface and intracellular NLRs**. In the absence of infection, the intracellular NLRs exist in an autoinhibited states. Upon recognition of the viral elicitor, the NLRs change to active conformations. Cell surface NLRs trigger signaling cascade sending factors into the nucleus to regulate gene expression. Some intracellular NLRs move to the nucleus to regulate defense gene expression. (Courtesy of Savithramma Dinesh-Kumar. Modified from Meier N, Hatch C, Nagalakshmi U, et al. Perspectives on intracellular perception of plant viruses. *Mol Plant Pathol*. 2019;20(9):1185-1190. Reprinted by permission of John Wiley & Sons, Inc.)

The activation of plant NLRs arrests virus amplification through induction of a variety of defenses. Typically induced is a hypersensitive response (HR) associated with visible necrotic lesions caused by a form of programmed cell death (although NLR activation without HR induction can still lead to a resistance response). Activation of the N NLR results in translational repression of PVX (family *Alphaflexiviridae*) transcripts and their redistribution to protein bodies but does not shut down global host translation.[194] Translational arrest of PVX transcripts during potato Rx1 NB-LRR–induced response requires the nucleocytoplasmic redistribution of Rx1 and likely is a major component in the Rx1-mediated extreme resistance response.[242]

Several phytohormones (eg, SA and JA) and other signaling molecules like ROS are upregulated in the local leaf upon HR induction. SA stimulates genetic and physiological responses across signaling networks that regulate effective basal resistance and induced systemic acquired resistance for viral and nonviral pathogens.[28] Perception of pathogen effectors in ETI triggers strong downstream responses including Ca^{2+} influxes and activation of MAPKs. Increase in cytoplasmic Ca^{2+} concentration and phosphorylation of RbohD by calcium-dependent kinases (CDKs) contribute to ROS signaling.[230] Activation of MAPKs leads to production of ethylene, camalexin, and indole glucosinolates and to transcriptional and proteome reprogramming. mRNAs encoding signaling-related proteins are upregulated in the Arabidopsis translatome after NB-LRR RPM1 activation by *Pseudomonas* effector AvrRpm1, whereas production of many proteins related to primary metabolism and the key growth-regulator protein target of rapamycin kinase (TOR) were downregulated.[194] These results predict a central role for translation in plant immune reactions and a role for TOR in the growth to defense transition needed for immune reactions.

Pathogen-Associated Molecular Pattern Triggered Immunity

Pathogen-associated molecular pattern triggered immunity (PTI) is the first line of plant defense against pathogens. Pattern recognition receptors (PRRs), such as the transmembrane receptorlike kinases, nuclear shuttle protein (NSP)-interacting kinase 1 (NIK1), the BRASSINOSTEROID INSENSITIVE 1 (BRI1)-ASSOCIATED RECEPTOR KINASE 1 BAK1, and the somatic embryogenesis receptor kinase 1(Serk1), and their coreceptor kinases are involved in plant PTI responses against bacterial and fungal pathogens. PRRs sense and recognize pathogen-associated molecular patterns (PAMPs) or endogenous host signals released upon infection known as danger-associated molecular patterns (DAMPs). Typically, PTI results in ROS production, activation of MAP kinases, induction of defense genes, synthesis of ethylene and SA, and deposition of the polysaccharide callose.

Since PRRs are cell-surface–located receptors, viruses as intracellular pathogens were not anticipated to trigger PTI, but an increasing body of evidence from recent studies show that they have a role in antiviral defenses to RNA and DNA viruses.[145,220,248,251] Arabidopsis plants become more susceptible to three RNA viruses, namely oilseed rape mosaic virus (ORMV; family *Virgaviridae*), TMV (family *Virgaviridae*), and TCV (family *Tombusviridae*), in the absences of BAK1 receptor.[144] The marker PTI responses of nonviral PTI are also induced during viral PTI, which suggests common mechanisms. How extracellular PAMP perception occurs in intracellular virus infection is not yet well understood.

Plant viruses both trigger and suppress antiviral PTI responses. In addition to viral proteins, viral dsRNAs can serve as PAMPS in plants and

trigger typical PTI responses.[222] NIK1-mediated antiviral response, which is activated by begomovirus-derived nucleic acids, have both similarities with PTI-like responses and its own special characteristics. Upon activation by phosphorylation, NIK1 phosphorylates ribosomal large subunit protein 10 (RPL10). Phosphorylated RPL10 is transported into the nucleus, where it partners with an RPL10-interacting MYB transcription factor to repress ribosomal protein genes, which subsequently results in global suppression of translation (Fig. 18.9).[241,355] As a viral counter defense, begomovirus nuclear shuttle protein inhibits the NIK1-mediated antiviral signaling. The CP of plum pox virus (PPV; family *Potyviridae*) suppresses PTI.[204] The MP of CMV (family *Bromoviridae*) suppresses ROS produced by other PTI responses as can the VSR P6 protein of CaMV (family *Caulimoviridae*),[140,356] indicating that P6 has a role both in suppression of RNA silencing and innate immunity.

Unfolded Protein Response in Regulation of Virus Infection

The unfolded protein response (UPR) machinery is important for plant viruses that rely extensively on the ER network for translation, replication, and cell-to-cell movement. Activation of UPR ameliorates necrotic symptoms caused by ER stress and gives the virus more space and time to replicate. Three ER stress sensors that contribute to the outcome of viral infection regulate separate but combined signaling cascades.[296] The major sensor is the inositol-requiring enzyme IRE1a and its isoform IRE1b, which are type 1 transmembrane protein kinase/endoribonucleases that recognize potyvirus 6K2 or potexvirus TGB3 proteins. As a response, IRE endonucleolytic cleavage of bZIP60 mRNA occurs, leading to production of a truncated, active bZIP60 transcription factor. Two other plant specific sensors are transcription factors bZIP28 and bZIP17, which have transmembrane domains that are proteolytically cleaved in response to environmental stresses. Once activated, bZIP60, bZIP28, and bZIP17 induce expression of genes involved in managing ER stress including chaperones for protein folding, cell fate determination, and innate immunity. A model was proposed in which bZIP60 and bZIP28 coordinate or overlap in their ability to activate genes that support potexvirus infection, while bZIP60 and bZIP17 coordinate to suppress infection.[71] bZIP60 and bZIP28 appear to restrict potyvirus infection while bZIP17 does not play a role.

CPR5, which anchors near the nuclear pore complex and restricts nuclear entry of bZIP60 and bZIP28, is a crucial inhibitor of ETI and modulates SA-dependent UPR. CPR5 is also associated with the recessive rym1 resistance to RYMV (family Solemoviridae) and resistance to CaMV (family *Caulimoviridae*), pepper mild mottle virus (PMMV; family *Virgaviridae*), and tobacco mild green mosaic virus (TMGMV; family *Virgaviridae*). The heterotrimeric G protein β subunit regulates the loads of CMV and TuMV as well as the spread of necrosis in Arabidopsis plants. Additional ER-resident factors that are involved in cell death regulation, autophagy, and Ca^{2+} signals include Bax inhibitor-1 (BI-1), C-cell lymphoma-2 (Bcl-2)-associated athanogene 7 (BAG7), NAC089, and NAC103, and their activities are coordinated with UPR responses that represent a complex network of interactions impacting potexvirus and potyvirus infection in Arabidopsis.[296]

Plant Viruses and Insect Vectors

More than 75% of plant viruses are transmitted by insect vectors such as aphids, leafhoppers, planthoppers, trips, and whiteflies. As climate change and global warming continue, insect populations are expanding; the number of migrations is rising; and migration distances are growing. This has led to an increase in insect vector-borne viral diseases affecting both cultured and uncultured plants.[165,334] This section summarizes some intriguing facets of plant-virus-vector interactions.

Transmission of viruses by insect vectors can be classified as either nonpersistent or persistent. Viruses transmitted in a nonpersistent fashion include CMV (family *Bromoviridae*), CaMV (family *Caulimoviridae*), and potyviruses, which are acquired by aphids feeding on epidermal cells of the host plant and then must be transmitted in a short timespan (seconds to hours) to uninfected plants. In nonpersistent transmission, virions are loosely bound to insect stylets and are not internalized into the gut (Fig. 18.10).[217] In persistent transmission, insect vectors require extended feeding time (hours to days) on an infected plant and stylets must penetrate the phloem for acquisition of the virus. Virions are then internalized in the insect gut system before moving out into the salivary glands. BYDV (family *Solemoviridae*) is transmitted by aphids in a persistent fashion but does not replicate within the aphids. Some plant viruses enter and replicate in the insect cells in these tissues, thus blurring the boundaries between insect and plant viruses. Most of the viruses transmitted by planthoppers, leafhoppers, whiteflies, and thrips, such as rice stripe virus (RSV; family *Phenuiviridae*), rice black streaked dwarf virus (RBSDV; family *Geminiviridae*), rice dwarf virus (RDV; family *Reoviridae*), and TSWV (family *Tospoviridae*), belong to this category (Fig. 18.11).[308]

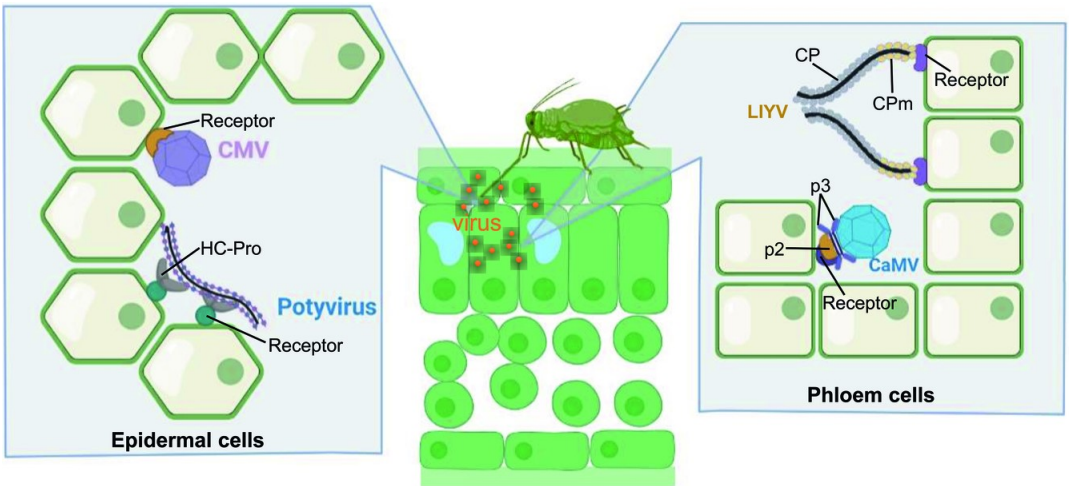

FIGURE 18.10 **Model depicting nonpersistent (*left*) and semipersistent (*right*) transmission of viruses by aphids**. Views in the *two black circles* are enlarged, showing the consequences of stylet penetrating the epidermis (*upper circle*) or phloem. Schematic diagram at *left* shows two strategies that nonpersistent viruses bind to aphid receptors. In the helper strategy, HC-Pro is indispensable for

binding between potyvirus CP and aphid receptors, which acts as a molecular bridge for potyvirus binding to the aphid stylet. In the capsid strategy, binding of CMV to vector receptors rely on amino acids in the CP directly. Diagram at *right* illustrates interactions between the sites in the vector's foregut and a semipersistent virus. CaMV binds to the insect's foregut with the help of viral-encoded protein p2 and p3. Two adjacent p3 can form a dimer on the surface of CaMV, with p2 helping the complex bind to the insect foregut. For lettuce infectious yellows crinivirus (LIYV), binding is mediated by the minor coat protein (CPm). (Figure is modified from Ng JCK, Falk BW. Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. *Annu Rev Phytopathol.* 2006;44:183-212.)

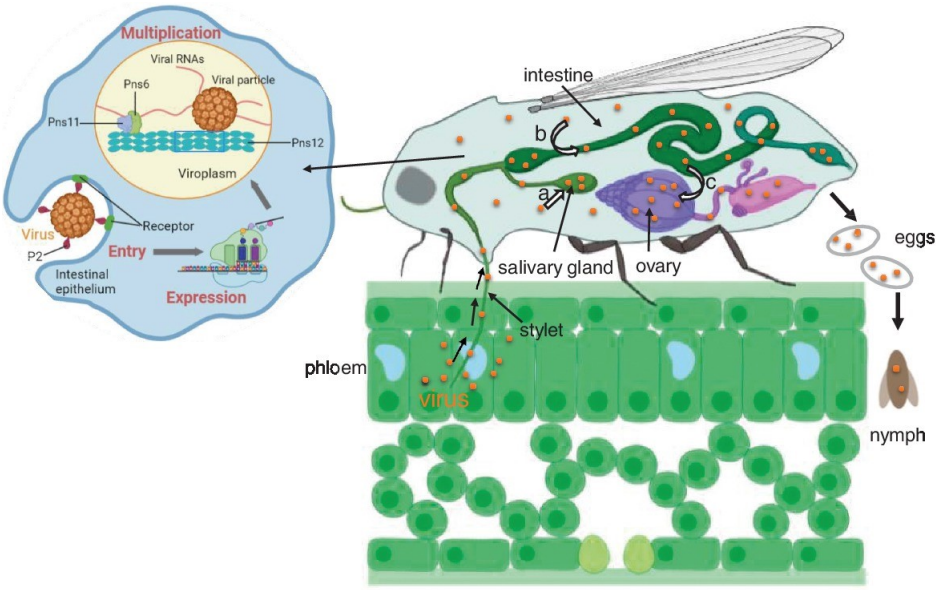

FIGURE 18.11 **Overview of infection routes of *Rice dwarf virus* (RDV) in its insect vector**. After the vector acquires RDV from infected plants, virions enter intestinal epithelium cells through outer capsid P2 protein-mediated membrane fusion and initiate replication cycles within insect cells. Letters (*a*, *b*, *c*) indicate three types of transmission barriers for RDV in the insect vectors: (*a*) salivary gland, (*b*) intestine, and (*c*) ovary, which are composed of different cell types and act as physical barriers to prevent viral infection. Diagram at left shows detailed replication cycle in insect cells. (Figure is modified from Wei T, Li Y. Rice reoviruses in insect vectors. *Annu Rev of Phytopathol.* 2016;54:99-120.)

Insect Vectors That Benefit From Feeding on Virus-Infected Plants

In a study published 70 years ago, the aphid population feeding on infected sugar beet plants grew faster than when fed on healthy plants.[128] Almost 40 years later, Fereres et al.[60] found that aphids [*Macrosiphum* (Sitobion) *avenae* (F)] feeding on BYDV-infected wheat had significantly shorter developmental times, greater fecundity, and a greater intrinsic rate of natural increase than aphids feeding on wheat that was virus free. More recent studies found that thrips (*Frankliniella occidentalis*) feeding on TSWV-infected pepper plants have greater juvenile survival and a shorter developmental rate, and invasive whiteflies (but not native whiteflies) feeding on plants infected with tobacco curly shoot virus (TbCSV; family *Geminiviridae*) or tomato yellow leaf curl China virus (TYLCCNV; family *Geminiviridae*) showed greater fecundity, longevity, and higher population density.[117] How these viruses increase insect vector fitness is not known, but evidence continues to accrue that coevolution between some viruses and their insect vectors can result in a mutualistic association.[28]

Viruses Modify Plant Metabolism to Enhance Transmission by Vectors

Phytohormones play essential roles regulating many aspects of a plant's life cycle, including seed germination, growth, development, responses to abiotic and biotic stress, and senescence. Phytohormones can also play a role in the plant's antiviral response as illustrated by infection of rice by RSV (family *Phenuiviridae*), whose CP enhances biosynthesis of JA, inducing a quicker and stronger antiviral response[308] (Fig. 18.12). To counter these antiviral properties, viruses also modulate phytohormone biosynthesis and signaling to benefit viral infection or the insect vector.[349] For example, the CMV 2b suppressor of RNA silencing suppresses JA-induced degradation of host jasmonate ZIM-domain proteins, thus increasing host attractiveness to insect vectors[315] (Fig. 18.12). JA is also reduced in TYLCCNV-infected tobacco plants by the virus ßC1 protein (also the suppressor of RNA silencing), making plants more attractive to B biotype whiteflies, thus increasing their population.[344] Virus infections can also modify the synthesis and/or signaling of other plant hormones such as SA, ethylene, and auxin.[349]

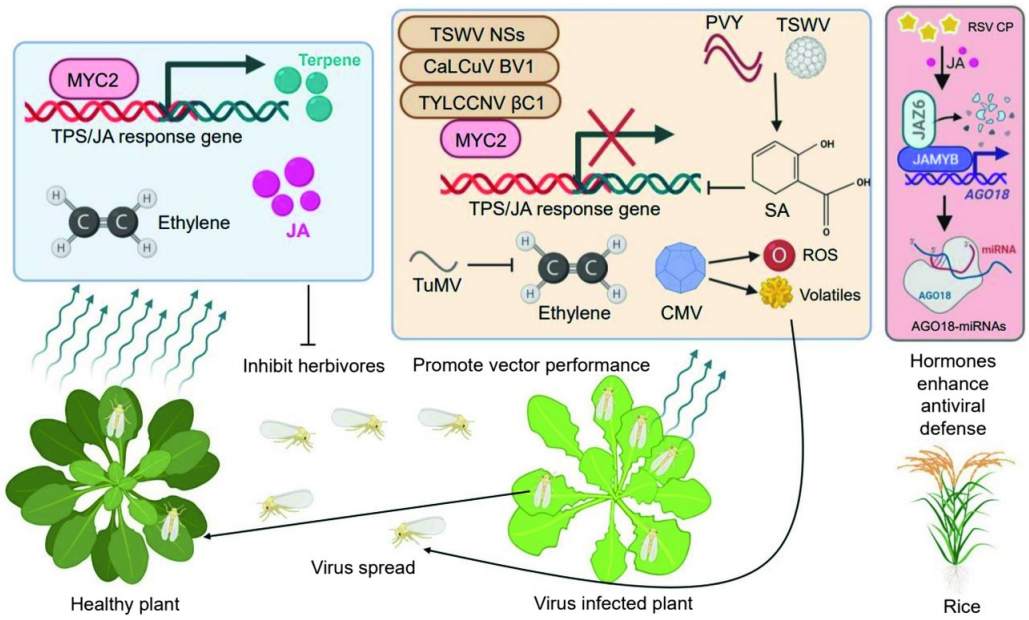

FIGURE 18.12 **Virus infection alters plant metabolism to promote insect vector performance**. Being a sessile organism, plants have evolved sophisticated mechanisms to manipulate plant hormones and volatiles to defend against insect herbivores. In healthy plants (*left panel*), feeding of herbivores actives JA signaling and other metabolic pathways. The virus can change the interplay between insect vectors and plants hormones/volatiles, thereby establishing a more attractive condition for their insect vectors (*middle and right panel*).

Guo et al.[83] found that WT CMV-infected tobacco plants produce higher concentrations of plant ROS than CMV that lacks the 2b gene (CMVΔ2b). Aphids feeding on WT CMV-infected tobacco plants engage in shorter probes and less phloem feeding compared with CMVΔ2b infected plants, which enhance virus acquisition and virus load, which is attributed to virus-induced accumulation of H_2O_2.

Other Virus-Induced Metabolic Modifications That Benefit the Vector

As described earlier, plants initiate sophisticated defenses when attacked by pests or viral infection. In addition to plant hormones and ROS, other signaling pathways also result in the production of defensive compounds. Terpenoid synthesis is induced in whitefly-infected plants but repressed in TYLCCV-infected plants by the ßC1 protein interacting with transcription factor MYC2.[174] This interaction suppresses activation of MYC2-regulated terpene synthase genes, thereby reducing whitefly resistance[166] (Fig. 18.12). In nonpersistent transmission, viruses like BYDV, potato leafroll virus, and CMV induce the production of plants volatiles to attract aphids. However, aphids with already acquired viruses are repelled by volatile cues released from viruses-infected host plants, leading them to feed on uninfected plants to enhance virus spread.[20,28,55,187]

In many virus-vector-host combinations, virus infection makes host plants more fit for insect colonization and in turn, the insect vector spreads the virus to new host plants. For aphid-CMV and leafhopper-RDV combinations, insect vector transmission is also important for filtering virus mutations and maintenance of genomic stability.[162,235] These intricate virus-insect-vector-host interactions, which can also be affected by environmental factors such as red-light[348] and involve symbiotic bacteria, are not always mutualistic, and many more studies are needed before a more complete understanding of these complex interactions is available.

Beneficial Plant Viruses

Plant Viruses That Benefit Their Hosts

Plant-virus interactions span the spectrum of parasitic to mutualistic.[245] Mutualism was recently recognized when some viruses were found to enhance the physiological performance of plants under abiotic stress, especially drought stress.[42] For example, BMV (family *Bromoviridae*), CMV (family *Bromoviridae*), TMV (family *Virgaviridae*), or tobacco rattle virus (TRV; family *Virgaviridae*) delay the onset of drought symptoms in *N. benthamiana* plants.[328] BMV also confers drought tolerance to rice plants, and CMV has the same affect in nine other host plants,[328] with changes linked to differences in host transcriptomes. Under normal watering conditions, CMV, PPV (family *Potyviridae*), and PVX (family *Alphaflexiviridae*) reduce plant growth and cause obvious foliar diseases. However, when water was deprived for 8 or more days, virus-infected plants showed better growth response than mock-treated plants.[2] The CMV 2b and the PVX TGB1 silencing suppressor proteins were implicated as important factors regulating stomatal conductance and transpiration, which were implicated in increased drought tolerance.[2,313] Interestingly, these conditional benefits were independent of antiviral gene silencing.

Drought tolerance in plants is regulated by a complex dynamic signaling network involving plant hormones SA, ABA, and JA. SA levels are elevated early in the infection by a wide range of plant viruses and contribute to induced systemic resistance to virus infection. ABA levels are induced by TMV, TuMV (family *Potyviridae*), CMV, BMV, TRV, and BaMV (family *Alphaflexiviridae*).[5,183,335] Combined elevation of SA and ABA can offset the hormones' normally antagonist roles impacting virus defenses.[4] The 2b and TGB1 proteins of CMV and PVX interfere with the balance of SA and ABA, which is linked to improving host tolerance to drought.[2,4] These studies are the key to gaining new understandings of disease, emergence of new pathogenic strains, and the evolution of plant defenses.[97]

Plant Viruses as Delivery Vehicles for siRNAs and Proteins

Both RNA and DNA plant viruses can serve as VIGS vectors, an RNA interference-based method for production of siRNAs that can control host gene expression and target other viruses, fungi, bacteria, nematodes, and insect pathogens without modifying the host genome.[16,149,173,257,352] VIGS can be used for any plant species following identification of a mild or asymptomatic virus that can infect the designated plant, and identifying suitable sites within the viral genome that can be disrupted by inserts without compromising infectivity of the virus. Inserts are commonly made between two ORFs in a virus that expresses its genome through synthesis of a polyprotein (eg, potyviruses) or by generation of a new sgRNA through manipulation of a sgRNA promoter (eg, PVX). VIGS vectors are usually delivered manually (by rub inoculation) or by agroinfiltration, a method where a cDNA copy of the (+)RNA virus genome is sandwiched between a strong plant promoter (usually the 35S promoter from CaMV) and a transcription termination sequence and added to a segment of the Ti plasmid of *Agrobacteria tumefaciens*, followed by syringe or vacuum infiltration of the bacteria into the plant. The bacteria then naturally excise and deliver a portion of the plasmid (the T-DNA) into a cell's cytoplasm, which eventually enters the nucleus where the viral genome is transcribed and then delivered into the cytoplasm where it is translated and replicated. Since viruses are naturally targets of RNA silencing, and the process (for RNA viruses) results in the generation of fully dsRNAs (by transitive silencing—described above) that are excellent templates for cellular dicer enzymes,[343] any additional sequence added to the viral genome will be in the siRNA population derived from the virus, which spread through the plant.

Prior to VIGS, downregulation of gene expression required the generation of transgenic plants, a process that can take years. With VIGS, transient downregulation of a gene of interest is achieved within weeks.[130] Viruses with wide host ranges, such as TRV and PVX, have become common tools in a plant molecular biologist's tool box for transient downregulation of host gene expression.[49] A recent important application of VIGS was for altering gene expression during the growing season in annual crops.[283] Viral genomes were delivered by either spraying virions or Agrobacteria carrying the viral genome directly onto annual crops to induce or repress flowering, change plant stature, alter drought sensitivity and fruit color, as well as other traits. The major drawback to VIGS vectors is the lack of stability of even short inserts, which currently preclude their use in long-lived trees and vines.

Plant Viruses and Biotechnology

Virions of plant viruses can be defined as biologically generated nanoscale structures with uniform physicochemical properties including icosahedral virions in the range of 30-70 nm and high-aspect-ratio rods and filaments of 300-2000 nm in length and 10-20 nm in width. Atomic resolution crystallographic and cryo-EM structures of plant viruses have paved the way for their development into nanobiotechnology devices.[269] Plant viral nanoparticles (VNPs) and empty viruslike particles (eVLPs) have been investigated as nanocarriers of a diverse number of reagents, including imaging compounds, immunomodulators, therapeutics, enzymes, and agrochemicals, for use in applications as nanoreactors, biosensors, and components in tissue engineering, batteries, photovoltaics, plasmonics, nanowires, transistors, and memory devices.[46,54,136,312]

The repetitive nature of CP subunits in virions provides a chemically uniform, multivalent platform for the selective loading of various active ingredients through genetic engineering, chemical bioconjugation, biomineralization, and encapsulation. Genetic engineering can be utilized both to modify surface-exposed amino acids and to fuse useful peptides and proteins that exist external to the virus particles.[26] The inner central channel and the surface of viruses can also be exploited as biotemplates for metal and semiconductor nanowire formation, with genetic modification of the surface to improve the affinity of different metals.[66] As examples of mineralized rod-shaped TMV (family *Virgoviridae*) templates, Au/Pd-TMV alloy nanowires have potential uses as high temperature resistors, hydrogen permeation membranes, multilayer capacitors, and magnetic memory storage materials,[168] and TMV-CdS nanowires with photocatalytic activities have been produced.[331] Encapsulation of cargo molecules to eVLPs is triggered by the surface charge, electrostatic interactions, and other binding properties of the CP sequences forming the internal surface of the eVLPs. The icosahedral cowpea chlorotic mottle virus (CCMV; family *Bromoviridae*) particle, 18 nm in diameter, has excellent properties for use as protein cages.[19] Disassembled CCMV CP subunits can reassemble under suitable pH conditions into eVLPs capable of encapsulating enzymes to form nanoreactors, as well as other types of molecules. In a recent study, nanodiamonds were encapsulated by CCMV CPs to be used as a tool in cell biology studies, given their excellent optical properties and high stability under physiological conditions.[324]

Ease and cost of production and purification affect the viability of nanobiotechnology platforms.[268] Examples of plant viruses that are frequently used for biotechnological applications include icosahedral cowpea mosaic virus (CPMV; family *Secoviridae*), flexuous rodlike structure PVX (family *Alphaflexiviridae*), or rigid rodlike shape TMV.[293] When produced via infection, these viruses are high yielding, but modifications to engineer VNPs are restricted to those that still retain infectivity of the virus. eVLPs have no such restrictions, and those produced from recombinant CPs expressed in bacteria, yeast, or in planta provide more flexibility than infection-derived VNPs. Expression of the CP and self-assembly into eVLPs is often effective with isometric plant viruses such as CPMV, where eVLPs can easily be produced in gram amounts in *N. benthamiana*.[250] *In vitro* assembly of viral CP with RNA operates in small-scale applications with TMV RNA, where the origin of assembly sequence (OAS) is sufficient to control capsid formation.[204] Skillful use of OAS allows for the construction of VLPs with the most imaginative shapes, such as nanoboomerangs and tetrapods.[53] A recent study showed that in planta production of eVLPs from papaya mosaic virus (PapMV; family *Alphaflexiviridae*) and alternanthera mosaic virus (AltMV; family *Alphaflexiviridae*) was more efficient when the CPs were expressed from a replicating PVX-based expression vector compared with expression from a nonreplicating vector, suggesting that replicating vectors make more effective scaffolds for VLP assembly.[279]

An asset of plant VNPs, especially in medical applications, is that they are noninfectious in mammals. Plant VNPs are currently in development as efficient generators of next generation of thermostable vaccines for regional fabrication and as immunomodulators with remarkable efficacy in animal models of various cancer types.[39] Examples of plant VNP-based approaches for *in vivo* medical applications include targeting of VNPs to tumors, VNPs for high-resolution imaging purposes, and VNPs for drug delivery.[54] Filamentous viruses are well suited for drug delivery and bioimaging applications because they can penetrate tissues and colonialize tumors efficiently.[263] A recent study comparing PVX and pepino mosaic virus (PepMV; family *Alphaflexiviridae*) filaments as VNPs showed that despite their similar sizes, they have different tumor homing properties and tissue specific distribution.[154] This indicates that full exploitation of the potential of plant VNPs for health applications requires that the optimal VNP is identified for each purpose.

Epilogue

Since the last edition of Field's Virology, there has been a revolution in our understanding of the complex relationships between plant viruses and hosts and we regret the limited amount of information that could be presented in this single chapter. Plant viruses are now envisioned as both exploitive of their hosts for replication and their insect vectors for transmission, and as existing in mutualistic associations for the benefit of one or both partners under certain environmental conditions. The arms race between viruses and their hosts has been further clarified with the addition of an ever-increasing number of co-opted proviral host factors leading the host to deploy similar proteins to disrupt these finely tuned processes. Increasing numbers of asymptomatic viruses are being identified from large scale sequencing studies and novel viruslike infectious agents are being discovered (e.g., ulaRNAs that use host MPs) that do not conform to the traditional definition of plant viruses. The likely linkage between replication and movement at MCS near PD has finally provided an explanation for one of the enduring mysteries of plant viruses: how MPs traffic cognate viruses exclusively without binding the gRNA specifically. Plant viruses are also increasingly finding usages in biotechnology, as manipulatable nanoparticles and as vectors for delivery of beneficial components into plants. While these remarkable advancements have been highlighted here, we also note that climate change and increasing cultivation of natural acreage are leading to the emergence of viruses that are threatening food security and the economies of countries overly dependent on a few crops. The continued lack of products for combatting virus infections in both annual crops and long-lived trees and vines cries out for researchers to convert their maturing understanding of host-virus-vector interactions into real life solutions (since genetic engineering of resistance into plants is unfortunately being rejected by growers and consumers). With bright minds entering the field, and with the availability of sufficient resources, we hope that these solutions will occupy a significant portion of the Plant Virus chapter in future editions of Field's Virology.

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REFERENCES

1. Agol VI, Gmyl AP. Emergency services of viral RNAs: repair and remodeling. Microbiol Mol Biol Rev. 2018;82:e00067-17.

2. Aguilar E, Cutrona C, del Toro FJ, et al. Virulence determines beneficial trade-offs in the response of virus-infected plants to drought via induction of salicylic acid. Plant Cell Environ. 2017;40:2909-2930.

3. Aguilar E, Gomez BG, Lozano-Duran R. Recent advances on the plant manipulation by geminiviruses. Curr Opin Plant Biol. 2020;56:56-64

4. Alazem M, He MH, Chang CH, et al. Disrupting the homeostasis of high mobility group protein promotes systemic movement of bamboo mosaic virus. Front Plant Sci. 2020;11:597665.

5. Alazem M, Lin K-Y, Lin N-S. The abscisic acid pathway has multifaceted effects on the accumulation of bamboo mosaic virus. Mol Plant Microbe Interact. 2014;27:177-189.

6. Ali Z, Abul-faraj A, Li LX, et al. Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system. Mol Plant. 2015;8:1288-1291.

7. Altabella T, Ramirez-Estrada K, Ferrer A. Phytosterol metabolism in plant positive-strand RNA virus replication. Plant Cell Rep. 2022;41:281-291.

8. Amari K, Lerich A, Schmitt-Keichinger C, et al. Tubule-guided cell-to-cell movement of a plant virus requires class XI myosin motors. PLoS Pathog. 2011;7:e1002327.

9. Amroun A, Priet S, de Lamballerie X, et al. Bunyaviridae RdRps: structure, motifs, and RNA synthesis machinery. Crit Rev Microbiol. 2017;43:753-778.

10. Arroyo-Mateos M, Sabarit B, Maio F, et al. Geminivirus replication protein impairs SUMO conjugation of proliferating cellular nuclear antigen at two acceptor sites. J Virol. 2018;92:e00611-18.

11. Baggs E, Dagdas G, Krasileva KV. NLR diversity, helpers and integrated domains: making sense of the NLR IDentity. Curr Opin Plant Biol. 2017;38:59-67.

12. Baliji S, Lacatus G, Sunter G. The interaction between geminivirus pathogenicity proteins and adenosine kinase leads to increased expression of primary cytokinin-responsive genes. Virology. 2010;402:238-247.

13. Baltes NJ, Voytas DF. Enabling plant synthetic biology through genome engineering. Trends Biotechnol. 2015;33:120-131.

14. Bamunusinghe D, Hemenway CL, Nelson RS, et al. Analysis of potato virus X replicase and TGBp3 subcellular locations. Virology. 2009;393:272-285.

15. Bao DR, Ganbaatar O, Cui XQ, et al. Down-regulation of genes coding for core RNAi components and disease resistance proteins via corresponding microRNAs might be correlated with successful soybean mosaic virus infection in soybean. Mol Plant Pathol. 2018;19:948-960.

16. Baulcombe DC. VIGS, HIGS and FIGS: small RNA silencing in the interactions of viruses or filamentous organisms with their plant hosts. Curr Opin Plant Biol. 2015;26:141-146.

17. Betti C, Lico C, Maffi D, et al. Potato virus X movement in Nicotiana benthamiana: new details revealed by chimeric coat protein variants. Mol Plant Pathol. 2012;13:198-203.

18. Bhardwaj U, Powell P, Goss DJ. Eukaryotic initiation factor (eIF) 3 mediates barley yellow dwarf viral mRNA 3′-5′ UTR interactions and 40S ribosomal subunit binding to facilitate cap-independent translation. Nucleic Acids Res. 2019;47:6225-6235.

19. Bode SA, Minten IJ, Nolte RJM, et al. Reactions inside nanoscale protein cages. Nanoscale. 2011;3:2376-2389.

20. Bosque-Perez NA, Eigenbrode SD. The influence of virus-induced changes in plants on aphid vectors: Insights from luteovirus pathosystems. Virus Res. 2011;159:201-205.

21. Brunkard JO, Runkel AM, Zambryski PC. The cytosol must flow: intercellular transport through plasmodesmata. Curr Opin Cell Biol. 2015;35:13-20.

22. Bujarski JJ. Genetic recombination in plant-infecting messenger-sense RNA viruses: overview and research perspectives. Front Plant Sci. 2013;4:68.

23. Butkovic A, Gonzalez R, Elena SF. Revisiting Orthotospovirus phylogeny using full-genome data and testing the contribution of selection, recombination and segment reassortment in the origin of members of new species. Arch Virol. 2021;166:491-499.

24. Cabanillas DG, Jiang J, Movahed N, et al. Turnip mosaic virus uses the SNARE protein VTI11 in an unconventional route for replication vesicle trafficking. Plant Cell. 2018;30:2594-2615.

25. Cao MJ, Du P, Wang XB, et al. Virus infection triggers widespread silencing of host genes by a distinct class of endogenous siRNAs in Arabidopsis. Proc Natl Acad Sci U S A. 2014;111:14613-14618.

26. Cardinale D, Carette N, Michon T. Virus scaffolds as enzyme nano-carriers. Trends Biotechnol. 2012;30:369-376.

27. Carpenter C, Simon A. Changes in locations of crossover sites over time in de novo generated RNA recombinants. Virology. 1996;223:165-173.

28. Carr JP, Murphy AM, Tungadi T, et al. Plant defense signals: players and pawns in plant-virus-vector interactions. Plant Sci. 2019;279:87-95.

29. Castillo-Gonzalez C, Liu X, Huang C, et al. Geminivirus-encoded TrAP suppressor inhibits the histone methyltransferase SUVH4/KYP to counter host defense. Elife. 2015;4:e06671.

30. Chandrasekaran M, Chun SC. Expression of PR-protein genes and induction of defense-related enzymes by Bacillus subtilis CBR05 in tomato (Solanum lycopersicum) plants challenged with Erwinia carotovora subsp carotovora. Biosci Biotechnol Biochem. 2016;80:2277-2283.

31. Chang CH, Hsu FC, Lee SC, et al. The nucleolar fibrillarin protein is required for helper virus-independent long-distance trafficking of a subviral satellite RNA in plants. Plant Cell. 2016;28:2586-2602.

32. Chen BH, Lin L, Lu YW, et al. Ubiquitin-like protein 5 interacts with the silencing suppressor p3 of rice stripe virus and mediates its degradation through the 26S proteasome pathway. PLoS Pathog. 2020;16:e1008780.

33. Chen H, Jackson D, Kim JY. Identification of evolutionarily conserved amino acid residues in homeodomain of KNOX proteins for intercellular trafficking. Plant Signal Behav. 2014;9:e28355.

34. Chen IH, Chen HT, Huang YP, et al. A thioredoxin NbTRXh2 from Nicotiana benthamiana negatively regulates the movement of bamboo mosaic virus. Mol Plant Pathol. 2018;19:405-417.

35. Chen XJ, Zhu M, Jiang L, et al. A multilayered regulatory mechanism for the autoinhibition and activation of a plant CC-NB-LRR resistance protein with an extra N-terminal domain. New Phytol. 2016;212:161-175.

36. Cheng XF, Wang AM. The potyvirus silencing suppressor protein VPg mediates degradation of SGS3 via ubiquitination and autophagy pathways. J Virol. 2016;91(1):e01478-16. https://doi.org/10.1128/JVI.01478-16

37. Cheng XF, Xiong RY, Li YZ, et al. Sumoylation of turnip mosaic virus RNA polymerase promotes viral infection by counteracting the host NPR1-mediated immune response. Plant Cell. 2017;29:508-525.

38. Chung BYW, Miller WA, Atkins JF, et al. An overlapping essential gene in the Potyviridae. Proc Natl Acad Sci U S A. 2008;105:5897-5902.

39. Chung YH, Church D, Koellhoffer EC, et al. Integrating plant molecular farming and materials research for next-generation vaccines. Nat Rev Mater. 2022;7(5):372-388. https://doi.org/10.1038/s41578-021-00399-5

40. Dai Z, He R, Bernards MA, Wang A. The cis-expression of the coat protein of turnip mosaic virus is essential for viral intercellular movement in plants. Mol Plant Pathol. 2020;21:1194-1211.

41. Das Sharma S, Kraft JJ, Miller WA, et al. Recruitment of the 40S ribosomal subunit to the 3′-untranslated region (UTR) of a viral mRNA, via the eIF4 complex, facilitates cap-independent translation. J Biol Chem. 2015;290:11268-11281.

42. Dastogeer KMG, Li H, Sivasithamparam K, et al. Fungal endophytes and a virus confer drought tolerance to Nicotiana bentharniana plants through modulating osmolytes, antioxidant enzymes and expression of host drought responsive genes. Environ Exp Bot. 2018;149:95-108.

43. De S, Pollari M, Varjosalo M, Makinen K. Association of host protein VARICOSE with HCPro within a multiprotein complex is crucial for RNA silencing suppression, translation, encapsidation and systemic spread of potato virus A infection. PLoS Pathog. 2020;16:e1008956.

44. den Hollander PW, Kieper SN, Borst JW, et al. The role of plasmodesma-located proteins in tubule-guided virus transport is limited to the plasmodesmata. Arch Virol. 2016;161:2431-2440.

45. Deom CM, Oliver MJ, Beachy RN. The 30-kilodalton gene-product of tobacco mosaic-virus potentiates virus movement. Science. 1987;237:389-394.

46. Dickmeis C, Kauth L, Commandeur U. From infection to healing: The use of plant viruses in bioactive hydrogels. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2021;13(1):e1662.

47. Dietrich C, Maiss E. Fluorescent labelling reveals spatial separation of potyvirus populations in mixed infected Nicotiana benthamiana plants. J Gen Virol. 2003;84:2871-2876.

48. Ding XS, Boydston CM, Nelson RS. Presence of brome mosaic virus in barley guttation fluid and its association with localized cell death response. Phytopathology. 2001;91:440-448.

49. Dommes AB, Gross T, Herbert DB, et al. Virus-induced gene silencing: empowering genetics in non-model organisms. J Exp Bot. 2019;70:757-770.

50. Dorokhov YL, Sheshukova EV, Byalik TE, et al. Diversity of plant virus movement proteins: what do they have in common? Processes. 2020;8:1547.

51. Du Z, Alekhina OM, Vassilenko KS, et al. Concerted action of two 3'' cap-independent translation enhancers increases the competitive strength of translated viral genomes. Nucleic Acids Res. 2017;45:9558-9572.

52. Dubiella U, Serrano I. The ubiquitin proteasome system as a double agent in plant-virus interactions. *Plants* (*Basel*). 2021;10:928.

53. Eber FJ, Eiben S, Jeske H, Wege C. RNA-controlled assembly of tobacco mosaic virus-derived complex structures: from nanoboomerangs to tetrapods. Nanoscale. 2015;7:344-355.

54. Eiben S, Koch C, Altintoprak K, et al. Plant virus-based materials for biomedical applications: trends and prospects. Adv Drug Deliv Rev. 2019;145:96-118.

55. Eigenbrode SD, Ding H, Shiel P, et al. Volatiles from potato plants infected with potato leafroll virus attract and arrest the virus vector, Myzus persicae (Homoptera: Aphididae). Proc Biol Sci. 2002;269:455-460.

56. Fatyol K, Ludman M, Burgyan J. Functional dissection of a plant Argonaute. Nucleic Acids Res. 2016;44:1384-1397.

57. Feng MF, Cheng RX, Chen ML, et al. Rescue of tomato spotted wilt virus entirely from complementary DNA clones. Proc Natl Acad Sci U S A. 2020;117:1181-1190.

58. Feng MF, Li LY, Cheng RX, et al. Development of a mini-replicon-based reverse-genetics system for rice stripe tenuivirus. J Virol. 2021;95:e00589-21.

59. Feng ZK, Inaba JI, Nagy PD. Tombusviruses target a major crossroad in the endocytic and recycling pathways via co-opting Rab7 small GTPase. J Virol. 2021;95:e0107621.

60. Fereres A, Lister RM, Araya JE, et al. Development and reproduction of the english grain aphid (homoptera, aphididae) on wheat cultivars infected with barley yellow dwarf virus. Environ Entomol. 1989;18:388-393.

61. Fernandez-Calvino L, Faulkner C, Walshaw J, et al. Arabidopsis plasmodesmal proteome. PLoS One. 2011;6:e18880.

62. Ferron F, Weber F, de la Torre JC, et al. Transcription and replication mechanisms of Bunyaviridae and Arenaviridae L proteins. Virus Res. 2017;234:118-134.

63. Fieulaine S, Witte MD, Theile CS, et al. Turnip yellow mosaic virus protease binds ubiquitin suboptimally to fine-tune its deubiquitinase activity. J Biol Chem. 2020;295:13769-13783.

64. Finch LK, Ling R, Napthine S, et al. Characterization of ribosomal frameshifting in theiler's murine encephalomyelitis virus. J Virol. 2015;89:8580-9858.

65. Folimonova SY, Tilsner J. Hitchhikers, highway tolls and roadworks: the interactions of plant viruses with the phloem. Curr Opin Plant Biol. 2018;43:82-88.

66. Freer AS, Guarnaccio L, Watford K, et al. SAXS characterization of genetically engineered tobacco mosaic virus nanorods coated with palladium in the absence of external reducing agents. J Colloid Interface Sci. 2013;392:213-218.

67. Fu S, Xu Y, Li CY, et al. Rice stripe virus interferes with s-acylation of remorin and induces its autophagic degradation to facilitate virus infection. Mol Plant. 2018;11:269-287.

68. Gallo A, Valli A, Calvo M, et al. A functional link between RNA replication and virion assembly in the potyvirus plum pox virus. J Virol. 2018;92:e02179-17.

69. Gao F, Simon AE. Differential use of 3'CITEs by the subgenomic RNA of Pea enation mosaic virus 2. Virology. 2017;510:194-204.

70. Garcia-Ruiz H, Carbonell A, Hoyer JS, et al. Roles and programming of Arabidopsis argonaute proteins during turnip mosaic virus infection. PLoS Pathog. 2015;11:e1004755.

71. Gayral M, Gaguancela OA, Vasquez E, et al. Multiple ER-to-nucleus stress signaling pathways are activated during plantago asiatica mosaic virus and turnip mosaic virusinfection in Arabidopsis thaliana. Plant J. 2020;103:1233-1245.

72. Geng C, Cong QQ, Li XD, et al. Developmentally regulated plasma membrane protein of Nicotiana benthamiana contributes to potyvirus

movement and transports to plasmodesmata via the early secretory pathway and the actomyosin system. Plant Physiol. 2015;167:394-410.

73. Geng GW, Wang DY, Liu ZF, et al. Translation of plant RNA viruses. Viruses. 2021;13:2499.

74. Geng GW, Yu CM, Li XD, et al. A unique internal ribosome entry site representing a dynamic equilibrium state of RNA tertiary structure in the 5′-UTR of wheat yellow mosaic virus RNA1. Nucleic Acids Res. 2020;48:390-404.

75. Genoves A, Navarro JA, Pallas V. The intra- and intercellular movement of melon necrotic spot virus (MNSV) depends on an active secretory pathway. Mol Plant Microbe Interact. 2010;23:263-272.

76. Ghosh D, Malavika M, Chakraborty S. Impact of viral silencing suppressors on plant viral synergism: a global agro-economic concern. Appl Microbiol Biotechnol. 2021;105: 6301-6313.

77. Ghoshal B, Sanfacon H. Temperature-dependent symptom recovery in Nicotiana benthamiana plants infected with tomato ringspot virus is associated with reduced translation of viral RNA2 and requires ARGONAUTE 1. Virology. 2014;456:188-197.

78. Gibbs AJ, Hajizadeh M, Ohshima K, et al. The potyviruses: an evolutionary synthesis is emerging. Viruses. 2020;12(2):132.

79. Gomez BG, Rosas-Diaz T, Shi CN, et al. The viral silencing suppressor P19 interacts with the receptor-like kinases BAM1 and BAM2 and suppresses the cell-to-cell movement of RNA silencing independently of its ability to bind sRNA. New Phytol. 2021;229:1840-1843.

80. Grangeon R, Jiang J, Wan J, et al. 6K(2)-induced vesicles can move cell to cell during turnip mosaic virus infection. Front Microbiol. 2013;4:351.

81. Gray SM, Power AG. Anthropogenic influences on emergence of vector-borne plant viruses: the persistent problem of potato virus Y. Curr Opin Virol. 2018;33:177-183.

82. Guerrero J, Regedanz E, Lu L, et al. Manipulation of the plant host by the geminivirus ac2/c2 protein, a central player in the infection cycle. Front Plant Sci. 2020;11:591. https://doi.org/10.3389/fpls.2020.00591

83. Guo HJ, Gu LY, Liu FQ, et al. Aphid-borne viral spread is enhanced by virus-induced accumulation of plant reactive oxygen species. Plant Physiol. 2019;179:143-155.

84. Guo Y, Liu BC, Ding ZZ, et al. Distinct mechanism for the formation of the ribonucleoprotein complex of tomato spotted wilt virus. J Virol. 2017;91:e00892-17.

85. Guo ZX, Lu JF, Wang XB, et al. Lipid flippases promote antiviral silencing and the biogenesis of viral and host siRNAs in Arabidopsis. Proc Natl Acad Sci U S A. 2017;114:1377-1382.

86. Guo ZX, Wang XB, Wang Y, et al. Identification of a new host factor required for antiviral RNAi and amplification of viral siRNAs. Plant Physiol. 2018;76:1587-1597.

87. Gupta N, Reddy K, Bhattacharyya D, et al. Plant responses to geminivirus infection: guardians of the plant immunity. Virol J. 2021;18:143.

88. Hafren A, Lohmus A, Makinen K. Formation of potato virus a-induced RNA granules and viral translation are interrelated processes required for optimal virus accumulation. PLoS Pathog. 2015;11:e1005314.

89. Hafren A, Macia JL, Love AJ, et al. Selective autophagy limits cauliflower mosaic virus infection by NBR1-mediated targeting of viral capsid protein and particles. Proc Natl Acad Sci U S A. 2017;114:e2026-e2035.

90. Hafren A, Ustun S, Hochmuth A, et al. Turnip mosaic virus counteracts selective autophagy of the viral silencing suppressor HCpro. Plant Physiol. 2018;176:649-662.

91. Hak H, Spiegelman Z. The tomato brown rugose fruit virus movement protein overcomes Tm-2(2) resistance in tomato while attenuating viral transport. Mol Plant Microbe Interact. 2021;34:1024-1032.

92. Hanley-Bowdoin L, Bejarano ER, Robertson D, et al. Geminiviruses: masters at redirecting and reprogramming plant processes. Nat Rev Microbiol. 2013;11:777-788.

93. Haxim Y, Ismayil A, Jia Q, et al. Autophagy functions as an antiviral mechanism against geminiviruses in plants. Elife. 2017;6:e23897.

94. Heinlein M. Plant virus replication and movement. Virology. 2015;479:657-671.

95. Helderman TA, Deurhof L, Bertran A, et al. An isoform of the eukaryotic translation elongation factor 1a (eEF1a) acts as a pro-viral factor required for tomato spotted wilt virus disease in Nicotiana benthamiana. Viruses. 2021;13:2190.

96. Helderman TA, Deurhof L, Bertran A, et al. Members of the ribosomal protein S6 (RPS6) family act as pro-viral factor for tomato spotted wilt orthotospovirus infectivity in Nicotiana benthamiana. Mol Plant Pathol. 2022;23:431-446.

97. Hily J-M, Poulicard N, Mora M-A, et al. Environment and host genotype determine the outcome of a plant-virus interaction: from antagonism to mutualism. New Phytol. 2016;209:812-822.

98. Hipper C, Brault V, Ziegler-Graff V, et al. Viral and cellular factors involved in phloem transport of plant viruses. Front Plant Sci. 2013;4:154.

99. Hofius D, Lie L, Hafren A, Coll NS. Autophagy as an emerging arena for plant-pathogen interactions. Curr Opin Plant Biol. 2017;38:117-123.

100. Hu T, Huang CJ, He YT, et al. βC1 protein encoded in geminivirus satellite concertedly targets MKK2 and MPK4 to counter host defense.

PLoS Pathog. 2019;15:e1007728.

101. Huang YP, Chen JS, Hsu YH, et al. A putative Rab-GTPase activation protein from Nicotiana benthamiana is important for bamboo mosaic virus intercellular movement. Virology. 2013;447:292-299.

102. Hung CJ, Huang YW, Liou MR et al. Phosphorylation of coat protein by protein kinase CK2 regulates cell-to-cell movement of bamboo mosaic virus through modulating RNA binding. Mol Plant Microbe Interact. 2014;27:1211-1225.

103. Hyodo K. Identification and characterization of host factors involved in plant RNA virus replication. J Gen Plant Pathol. 2021;87:415-417.

104. Hyodo K, Hashimoto K, Kuchitsu K, et al. Harnessing host ROS-generating machinery for the robust genome replication of a plant RNA virus. Proc Natl Acad Sci U S A. 2017;114:E1282-E1290.

105. Hyodo K, Okuno T. Hijacking of host cellular components as proviral factors by plant-infecting viruses. Adv Virus Res. 2020;107:37-86.

106. Ilyas M, Du Z, Simon A. Opium poppy mosaic virus has an Xrn-resistant, translated subgenomic RNA and a BTE 3′ CITE. J Virol. 2021;95:e02109-e02120.

107. Iqbal Z, Iqbal MS, Singh SP, et al. Ca2+/calmodulin complex triggers CAMTA transcriptional machinery under stress in plants: signaling cascade and molecular regulation. Front Plant Sci. 2020;11:598327.

108. Ishibashi K, Matsumoto-Yokoyama E, et al. A tomato spotted wilt virus S RNA-based replicon system in yeast. Sci Rep. 2017;7:12647.

109. Ivanov KI, Eskelin K, Basic M, et al. Molecular insights into the function of the viral RNA silencing suppressor HCPro. Plant J. 2016;85:30-45.

110. Iwakawa H, Tomari Y. Molecular insights into microRNA-mediated translational repression in plants. Mol Cell. 2013;52:591-601.

111. Jackson AO, Dietzgen RG, Goodin MM, et al. Development of model systems for plant rhabdovirus research. Adv Virus Res. 2018;102:23-57.

112. Jaramillo-Mesa H, Gannon M, Holshbach E, et al. The triticum mosaic virus internal ribosome entry site relies on a picornavirus-like YX-AUG motif to designate the preferred translation initiation site and to likely target the 18S rRNA. J Virol. 2019;93:e01705-e01718.

113. Jiang DM, Sano T, Tsuji M, et al. Comprehensive diversity analysis of viroids infecting grapevine in China and Japan. Virus Res. 2012;169:237-245.

114. Jiang DM, Wang M, Li SF. Functional analysis of a viroid RNA motif mediating cell-to-cell movement in Nicotiana benthamiana. J Gen Virol. 2017;98:121-125.

115. Jiang J, Patarroyo C, Cabanillas DG, et al. The vesicle-forming 6K(2) protein of turnip mosaic virus interacts with the COPII coatomer Sec24a for viral systemic infection. J Virol. 2015;89:6695-6710.

116. Jiang LL, Lu YW, Zheng XY, et al. The plant protein NbP3IP directs degradation ofRice stripe virusp3 silencing suppressor protein to limit virus infection through interaction with the autophagy-related protein NbATG8. New Phytol. 2021;229:1036-1051.

117. Jiu M, Zhou XP, Tong L, et al. Vector-virus mutualism accelerates population increase of an invasive whitefly. PLoS One. 2007;2:e182.

118. Johnson PZ, Kasprzak WK, Shapiro BA, et al. Structural characterization of a new subclass of panicum mosaic virus-like 3′ cap-independent translation enhancer. Nucleic Acids Res. 2022;50:1601-1619.

119. Jones RAC, Naidu RA. Global dimensions of plant virus diseases: current status and future perspectives. Annu Rev Virol. 2019;6:387-409.

120. Ju HJ, Samuels TD, Wang YS, et al. The potato virus X TGBp2 movement protein associates with endoplasmic reticulum-derived vesicles during virus infection. Plant Physiol. 2005;138:1877-1895.

121. Kappagantu M, Collum TD, Dardick C, et al. Viral hacks of the plant vasculature: the role of phloem alterations in systemic virus infection. Annu Rev Virol. 2020;7:351-370.

122. Karetnikov A, Lehto K. The RNA2 5′ leader of Blackcurrant reversion virus mediates efficient in vivo translation through an internal ribosomal entry site mechanism. J Gen Virol. 2007;88:286-297.

123. Karetnikov A, Lehto K. Translation mechanisms involving long-distance base pairing interactions between the 5′ and 3′ non-translated regions and internal ribosomal entry are conserved for both genomic RNAs of Blackcurrant reversion nepovirus. Virology. 2008;371:292-308.

124. Kasteel DTJ, Perbal MC, Boyer JC, et al. The movement proteins of cowpea mosaic virus and cauliflower mosaic virus induce tubular structures in plant and insect cells. J Gen Virol. 1996;77:2857-2864.

125. Kawaguchi-Ito Y, Li SF, Tagawa M, et al. Cultivated grapevines represent a symptomless reservoir for the transmission of hop stunt viroid to hop crops: 15 years of evolutionary analysis. PLoS One. 2009;4:e8386.

126. Kawakubo S, Gao FL, Li SF, et al. Genomic analysis of the brassica pathogen turnip mosaic potyvirus reveals its spread along the former trade routes of the Silk Road. Proc Natl Acad Sci U S A. 2021;118:e2021221118.

127. Kehr J, Kragler F. Long distance RNA movement. New Phytol. 2018;218:29-40.

128. Kennedy JS. Benefits to aphids from feeding on galled and virus-infected leaves. Nature. 1951;168:825-826.

129. Kerenyi Z, Merai Z, Hiripi L, et al. Inter-kingdom conservation of mechanism of nonsense-mediated mRNA decay. EMBO J. 2008;27:1585-1595.

130. Khakhar A, Voytas DF. RNA viral vectors for accelerating plant synthetic biology. Front Plant Sci. 2021;12:668580.

131. Khan M, Subramaniam R, Desveaux D. Of guards, decoys, baits and traps: pathogen perception in plants by type III effector sensors. Curr Opin Microbiol. 2016;29:49-55.

132. Kim SH, MacFarlane S, Kalinina NO, et al. Interaction of a plant virus-encoded protein with the major nucleolar protein fibrillarin is required for systemic virus infection. Proc Natl Acad Sci U S A. 2007;104:11115-11120.

133. Kim YS, An CF, Park S, et al. CAMTA-mediated regulation of salicylic acid immunity pathway genes in arabidopsis exposed to low temperature and pathogen infection. Plant Cell. 2017;29:2465-2477.

134. Kimura S, Waszczak C, Hunter K, et al. Bound by fate: the role of reactive oxygen species in receptor-like kinase signaling. Plant Cell. 2017;29:638-654.

135. Kitchen A, Shackelton LA, Holmes EC. Family level phylogenies reveal modes of macroevolution in RNA viruses. Proc Natl Acad Sci U S A. 2011;108:238-243.

136. Koch C, Eber FJ, Azucena C, et al. Novel roles for well-known players: from tobacco mosaic virus pests to enzymatically active assemblies. Beilstein J Nanotechnol. 2016;7:613-629.

137. Kockler ZW, Gordenin DA. From RNA world to SARS-CoV-2: the edited story of RNA viral evolution. Cells. 2021;10:1557.

138. Kolondam B, Rao P, Sztuba-Solinska J, et al. Co-infection with two strains of Brome mosaic bromovirus reveals common RNA recombination sites in different hosts. Virus Evol. 2015;1(1):vev021.

139. Komoda K, Ishibashi K, Kawamura-Nagaya K, et al. Possible involvement of eEF1A in tomato spotted wilt virus RNA synthesis. Virology. 2014;468:81-87.

140. Kong J, Wei MS, Li GF, et al. The cucumber mosaic virus movement protein suppresses PAMP-triggered immune responses in Arabidopsis and tobacco. Biochem Biophys Res Commun. 2018;498:395-401.

141. Koonin EV, Krupovic M, Ishino S, et al. The replication machinery of LUCA: common origin of DNA replication and transcription. BMC Biol. 2020;18:61.

142. Kormelink R, Garcia ML, Goodin M, et al. Negative-strand RNA viruses: the plant-infecting counterparts. Virus Res. 2011;162:184-202.

143. Kormelink R, Verchot J, Tao XR, et al. The Bunyavirales: the plant-infecting counterparts. Viruses. 2021;13:842.

144. Korner CJ, Klauser D, Niehl, A, et al. The immunity regulator BAK1 contributes to resistance against diverse RNA viruses. Mol Plant Microbe Interact. 2013;26:1271-1280.

145. Korner CJ, Pitzalis N, Pena EJ, et al. Crosstalk between PTGS and TGS pathways in natural antiviral immunity and disease recovery. Nat Plants. 2018;4:157-164.

146. Kovalev N, Pogany J, Nagy PD. Interviral recombination between plant, insect, and fungal RNA viruses: role of the intracellular Ca2+/Mn2+ pump. J Virol. 2020;94:e01015-19.

147. Krishnamurthy K, Heppler M, Mitra R, et al. The Potato virus X TGBp3 protein associates with the ER network for virus cell-to-cell movement. Virology. 2003;309:135-151.

148. Kroj T, Chanclud E, Michel-Romiti C, et al. Integration of decoy domains derived from protein targets of pathogen effectors into plant immune receptors is widespread. New Phytol. 2016;210:618-626.

149. Kumar DR, Saini T, Anandalakshmi R. Virus-induced gene silencing (VIGS) and its applications. In: Patil BL, ed. *Genes, Genetics and Transgenics for Virus Resistance in Plants*. Caister Academic Press; 2018:277-327.

150. Kushwaha NK, Hafrn A, Hofius D. Autophagy-virus interplay in plants: from antiviral recognition to proviral manipulation. Mol Plant Pathol. 2019;20:1211-1216.

151. Kwon SJ, Bodaghi S, Dang T, et al. Complete nucleotide sequence, genome organization, and comparative genomic analyses of citrus yellow-vein associated virus (CYVaV). Front Microbiol. 2021;12:683130.

152. Lai JB, Chen H, Teng KL, et al. RKP, a RING finger E3 ligase induced by BSCTV C4 protein, affects geminivirus infection by regulation of the plant cell cycle. Plant J. 2009;57:905-917.

153. Lazareva EA, Lezzhov AA, Golyshev SA, et al. Similarities in intracellular transport of plant viral movement proteins BMB2 and TGB3. J Gen Virol. 2017;98:2379-2391.

154. Le DHT, Mendez-Lopez E, Wang C, et al. Biodistribution of filamentous plant virus nanoparticles: pepino mosaic virus versus potato virus X. Biomacromolecules. 2019;20:469-477.

155. Le MT, Kasprzak WK, Kim T, et al. Folding behavior of a T-shaped, ribosome-binding translation enhancer implicated in a wide-spread conformational switch. Elife. 2017;6:e22883.

156. Lee JY. Plasmodesmata: a signaling hub at the cellular boundary. Curr Opin Plant Biol. 2015;27:133-140.

157. Levy A, Tilsner J. Creating contacts between replication and movement at plasmodesmata—a role for membrane contact sites in plant virus infections? Front Plant Sci. 2020;11:862.

158. Levy A, Zheng JY, Lazarowitzl SG. Synaptotagmin SYTA forms er-plasma membrane junctions that are recruited to plasmodesmata for plant virus movement. Curr Biol. 2015;25:2018-2025.

159. Lezzhov AA, Atabekova AK, Tolstyko EA, et al. RNA phloem transport mediated by pre-miRNA and viral tRNA-like structures. Plant Sci. 2019;284:99-107.

160. Li FF, Wang AM. RNA decay is an antiviral defense in plants that is counteracted by viral RNA silencing suppressors. PLoS Pathog. 2018;14:e1007228.

161. Li FF, Zhang CW, Li YZ, et al. Beclin1 restricts RNA virus infection in plants through suppression and degradation of the viral polymerase. Nat Commun. 2018;9:1268.

162. Li HY, Roossinck MJ. Genetic bottlenecks reduce population variation in an experimental RNA virus population. J Virol. 2004;78:10582-10587.

163. Li J, Huang HN, Zhu M, et al. A plant immune receptor adopts a two-step recognition mechanism to enhance viral effector perception. Mol Plant. 2019;12:248-262.

164. Li J, Xin J, Zhao XY, et al. Expression, purification and crystallization of the N-terminal Solanaceae domain of the Sw-5b NLR immune receptor. Acta Crystallogr F Struct Biol Commun. 2021;77:8-12.

165. Li P, Shu YN, Fu S, et al. Vector and nonvector insect feeding reduces subsequent plant susceptibility to virus transmission. New Phytol. 2017;215:699-710.

166. Li R, Weldegergis BT, Li J, et al. Virulence factors of geminivirus interact with myc2 to subvert plant resistance and promote vector performance. Plant Cell. 2014;26:4991-5008.

167. Li YY, Tan GL, Xiao L, et al. A multiyear survey and identification of pepper- and tomato-infecting viruses in Yunnan Province, China. Front Microbiol. 2021;12:623875.

168. Lim J-S, Kim S-M, Lee S-Y, et al. Formation of Au/Pd alloy nanoparticles on TMV. J Nanomater. 2010;2010:620505.

169. Linnik O, Liesche J, Tilsner J, et al. Unraveling the structure of viral replication complexes at super-resolution. Front Plant Sci. 2013;4:6.

170. Liu JY, Carino E, Bera S, et al. Structural analysis and whole genome mapping of a new type of plant virus subviral RNA: umbravirus-like associated RNAs. Viruses. 2021;13:646.

171. Liu JY, Simon AE. Identification of novel 5′ and 3′ translation enhancers in umbravirus-like coat protein-deficient RNA replicons. J Virol. 2022;96(7):e0173621.

172. Lohmus A, Varjosalo M, Makinen K. Protein composition of 6K2-induced membrane structures formed during Potato virus A infection. Mol Plant Pathol. 2016;17:943-958.

173. Lu R, Martin-Hernandez AM, Peart JR, et al. Virus-induced gene silencing in plants. Methods. 2003;30:296-303.

174. Luan JB, Yao DM, Zhang T, et al. Suppression of terpenoid synthesis in plants by a virus promotes its mutualism with vectors. Ecol Lett. 2013;16:390-398.

175. Lucas WJ, Lee JY. Plant cell biology—plasmodesmata as a supracellular control network in plants. Nat Rev Mol Cell Biol. 2004;5:712-726.

176. Lukhovitskaya NI, Cowan GH, Vetukuri RR, et al. Importin-α-mediated nucleolar localization of potato mop-top virus TRIPLE GENE BLOCK1 (TGB1) protein facilitates virus systemic movement, whereas TGB1 self-interaction is required for cell-to-cell movement in Nicotiana benthamiana. Plant Physiol. 2015;167:738-752.

177. Luria N, Smith E, Reingold V, et al. A new Israeli tobamovirus isolate infects tomato plants harboring Tm-2(2) resistance genes. PLoS One. 2017;12:e0170429.

178. Lv MF, Xie L, Song XJ, et al. Phloem-limited reoviruses universally induce sieve element hyperplasia and more flexible gateways, providing more channels for their movement in plants. Sci Rep. 2017;7:16467.

179. Ma XF, Zhou YJ, Moffett P. Alterations in cellular RNA decapping dynamics affect tomato spotted wilt virus cap snatching and infection in Arabidopsis. New Phytol. 2019;224:789-803.

180. Maina S, Barbetti MJ, Edwards OR, et al. Genetic connectivity between papaya ringspot virus genomes from Papua New Guinea and Northern Australia, and new recombination insights. Plant Dis. 2019;103:737-747.

181. Makinen K, Lohmus A, Pollari M. Plant RNA regulatory network and rna granules in virus infection. Front Plant Sci. 2017;8:2093.

182. Manabayeva SA, Shamekova M, Park JW, et al. Differential requirements for Tombusvirus coat protein and P19 in plants following leaf versus root inoculation. Virology. 2013;439:89-96.

183. Manacorda CA, Gudesblat G, Sutka M, et al. TuMV triggers stomatal closure but reduces drought tolerance in Arabidopsis. Plant Cell Environ. 2021;44:1399-1416.

184. Martinez-Perez M, Aparicio F, Lopez-Gresa MP, et al. Arabidopsis m(6)A demethylase activity modulates viral infection of a plant virus and

the m(6)A abundance in its genomic RNAs. Proc Natl Acad Sci U S A. 2017;114:10755-10760.

185. Martins LGC, Raimundo GAS, Ribeiro NGA, et al. A begomovirus nuclear shuttle protein-interacting immune hub: hijacking host transport activities and suppressing incompatible functions. Front Plant Sci. 2020;11:398.

186. Mascia T, Gallitelli D. Synergies and antagonisms in virus interactions. Plant Sci. 2016;252:176-192.

187. Mauck KE, De Moraes CM, Mescher MC. Deceptive chemical signals induced by a plant virus attract insect vectors to inferior hosts. Proc Natl Acad Sci U S A. 2010;107:3600-3605.

188. Maule AJ, Benitez-Alfonso Y, Faulkner C. Plasmodesmata—membrane tunnels with attitude. Curr Opin Plant Biol. 2011;14:683-690.

189. May J, Johnson P, Saleem H, et al. A sequence-independent, unstructured internal ribosome entry site is responsible for internal expression of the coat protein of turnip crinkle virus. J Virol. 2017;91:e02421-16.

190. May JP, Johnson PZ, Ilyas M, et al. The multifunctional long-distance movement protein of pea enation mosaic virus 2 protects viral and host transcripts from nonsense-mediated decay. MBio. 2020;11:e00204-20.

191. May JP, Simon AE. Targeting of viral RNAs by Upf1-mediated RNA decay pathways. Curr Opin Virol. 2021;47:1-8.

192. May JP, Yuan X, Sawicki E, et al. RNA virus evasion of nonsense-mediated decay. PLoS Pathog. 2018;14:e1007459.

193. Mestre P, Baulcombe DC. Elicitor-mediated oligomerization of the tobacco N disease resistance protein. Plant Cell. 2006;18:491-501.

194. Meteignier LV, Zhou J, Cohen M, et al. NB-LRR signaling induces translational repression of viral transcripts and the formation of RNA processing bodies through mechanisms differing from those activated by UV stress and RNAi. J Exp Bot. 2016;67:2353-2366.

195. Mingot A, Valli A, Rodamilans B, et al. The P1N-PISPO trans-frame gene of sweet potato feathery mottle potyvirus is produced during virus infection and functions as an RNA silencing suppressor. J Virol. 2016;90:3543-3557.

196. Miras M, Miller WA, Truniger V, Aranda MA. Non-canonical translation in plant RNA viruses. Front Plant Sci. 2017;8:494.

197. Miras M, Sempere RN, Kraft JJ, et al. Interfamilial recombination between viruses led to acquisition of a novel translation-enhancing RNA element that allows resistance breaking. New Phytol. 2014;202:233-246.

198. Mondal S, Ghanim M, Roberts A, et al. Different potato virus Y strains frequently co-localize in single epidermal leaf cells and in the aphid stylet. J Gen Virol. 2021;102(3).

199. Moreno AB, Lopez-Moya JJ. When viruses play team sports: mixed infections in plants. Phytopathology. 2020;110:29-48.

200. Moreno IM, Thompson JR, Garcia-Arenal F. Analysis of the systemic colonization of cucumber plants by cucumber green mottle mosaic virus. J Gen Virol. 2004;85:749-759.

201. Morozov SY, Solovyev AG. Small hydrophobic viral proteins involved in intercellular movement of diverse plant virus genomes. AIMS Microbiol. 2020;6:305-329.

202. Movahed N, Cabanillas DG, Wan J, et al. Turnip mosaic virus components are released into the extracellular space by vesicles in infected leaves. Plant Physiol. 2019;180:1375-1388.

203. Movahed N, Patarroyo C, Sun JQ, et al. Cylindrical inclusion protein of turnip mosaic virus serves as a docking point for the intercellular movement of viral replication vesicles. Plant Physiol. 2017;175:1732-1744.

204. Mueller A, Eber FJ, Azucena C, et al. Inducible site-selective bottom-up assembly of virus-derived nanotube arrays on RNA-equipped wafers. ACS Nano. 2011;5:4512-4520.

205. Mukhamedzhanova AA, Karpova OV, Rodionova NP, et al. Nonspecific activation of translation of encapsidated potexviral RNA with involvement of potato virus X movement protein TGB1. Dokl Biochem Biophys. 2009;428:239-2341.

206. Murphy AM, Zhou T, Carr JP. An update on salicylic acid biosynthesis, its induction and potential exploitation by plant viruses. Curr Opin Virol. 2020;42:8-17.

207. Mushegian AR, Koonin EV. Cell-to-cell movement of plant-viruses—insights from amino-acid-sequence comparisons of movement proteins and from analogies with cellular-transport systems. Arch Virol. 1993;133:239-257.

208. Nagy PD. Exploitation of a surrogate host, Saccharomyces cerevisiae, to identify cellular targets and develop novel antiviral approaches. Curr Opin Virol. 2017;26:132-140.

209. Nagy PD. Host protein chaperones, RNA helicases and the ubiquitin network highlight the arms race for resources between tombusviruses and their hosts. Adv Virus Res. 2020;107:133-158.

210. Nagy PD, Feng ZK. Tombusviruses orchestrate the host endomembrane system to create elaborate membranous replication organelles. Curr Opin Virol. 2021;48:30-41.

211. Nagy PD, Lin WW. Taking over cellular energy-metabolism for TBSV replication: the high ATP requirement of an RNA virus within the viral replication organelle. Viruses. 2020;12:56.

212. Nagy PD, Pogany J. The dependence of viral RNA replication on co-opted host factors. Nat Rev Microbiol. 2012;10:137-149.

213. Nagy PD, Pogany J, Xu K. Cell-free and cell-based approaches to explore the roles of host membranes and lipids in the formation of viral replication compartment induced by tombusviruses. Viruses. 2016;8:68.

214. Nakahara KS, Masuta C, Yamada S, et al. Tobacco calmodulin-like protein provides secondary defense by binding to and directing degradation of virus RNA silencing suppressors. Proc Natl Acad Sci U S A. 2012;109:10113-11018.

215. Navarro JA, Pallas V. An update on the intracellular and intercellular trafficking of carmoviruses. Front Plant Sci. 2017;8:1801.

216. Navas-Castillo J, Fiallo-Olive E, Sanchez-Campos S. Emerging virus diseases transmitted by whiteflies. Annu Rev Phytopathol. 2011;49:219-248.

217. Ng JCK, Falk BW. Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. Annu Rev Phytopathol. 2006;44:183-212.

218. Nguyen HD, Tomitaka Y, Ho SYW, et al. Turnip mosaic potyvirus probably first spread to eurasian brassica crops from wild orchids about 1000 years ago. PLoS One. 2013;8:e55336.

219. Nguyen-Dinh V, Herker E. Ultrastructural features of membranous replication organelles induced by positive-stranded RNA viruses. Cells. 2021;10:2407.

220. Nicaise V, Candresse T. Plum pox virus capsid protein suppresses plant pathogen-associated molecular pattern (PAMP)-triggered immunity. Mol Plant Pathol. 2017;18(6):878-886.

221. Niehl A, Pena EJ, Amari K, et al. Microtubules in viral replication and transport. Plant J. 2013;75:290-308.

222. Niehl A, Wyrsch I, Boller T, et al. Double-stranded RNAs induce a pattern-triggered immune signaling pathway in plants. New Phytol. 2016;211:1008-1019.

223. Nishikiori M, Ahlquist P. Transmembrane redox regulation of genome replication functions in positive-strand RNA viruses. Curr Opin Virol. 2021;47:25-31.

224. Ohshima K, Yamaguchi Y, Hirota R, et al. Molecular evolution of turnip mosaic virus: evidence of host adaptation, genetic recombination and geographical spread. J Gen Virol. 2002;83:1511-1521.

225. Oliver JE, Whitfield AE. The genus tospovirus: emerging bunyaviruses that threaten food security. Annu Rev Virol. 2016;3:101-124.

226. Opalka N, Brugidou C, Bonneau C, et al. Movement of rice yellow mottle virus between xylem cells through pit membranes. Proc Natl Acad Sci U S A. 1998;95:3323-3328.

227. Park S-H, Li F, Renaud J, et al. NbEXPA1, an alpha-expansin, is plasmodesmata-specific and a novel host factor for potyviral infection. Plant J. 2017;92:846-861.

228. Paudel DB, Sanfacon H. Mapping of sequences in the 5′ region and 3′ UTR of tomato ringspot virus RNA2 that facilitate cap-independent translation of reporter transcripts in vitro. PLoS One. 2021;16:e0249928.

229. Peng B, Liu LM, Wu HJ, et al. Interspecific recombination between zucchini tigre mosaic virus and papaya ringspot virus infecting cucurbits in China. Front Microbiol. 2021;12:773992.

230. Peng YJ, van Wersch R, Zhang YL. Convergent and divergent signaling in PAMP-triggered immunity and effector-triggered immunity. Mol Plant Microbe Interact. 2018;31:403-409.

231. Perraki A, Cacas JL, Crowet JM, et al. Plasma membrane localization of solanum tuberosum remorin from group 1, homolog 3 is mediated by conformational changes in a novel c-terminal anchor and required for the restriction of potato virus X movement. Plant Physiol. 2012;160:624-637.

232. Pollari M, De S, Wang A, et al. The potyviral silencing suppressor HCPro recruits and employs host ARGONAUTE1 in pro-viral functions. PLoS Pathog. 2020;16:e1008965.

233. Pouwels J, van der Velden T, Willemse J, et al. Studies on the origin and structure of tubules made by the movement protein of cowpea mosaic virus. J Gen Virol. 2004;85:3787-3796.

234. Prasanth KR, Kovalev N, Martin IFD, et al. Screening a yeast library of temperature-sensitive mutants reveals a role for actin in tombusvirus RNA recombination. Virology. 2016;489:233-242.

235. Pu YY, Kikuchi A, Moriyasu Y, et al. Rice dwarf viruses with dysfunctional genomes generated in plants are filtered out in vector insects: implications for the origin of the virus. J Virol. 2011;85:2975-2979.

236. Pyott DE, Sheehan E, Molnar A. Engineering of CRISPR/Cas9-mediated potyvirus resistance in transgene-free Arabidopsis plants. Mol Plant Pathol. 2016;17:1276-1288.

237. Qiao WJ, Medina V, Kuo YW, et al. A distinct, non-virion plant virus movement protein encoded by a crinivirus essential for systemic infection. Mbio. 2018;9:e02230-18. doi:10.1128/mBio.02230-18

238. Raffaele S, Bayer E, Lafarge D, et al. Remorin, a solanaceae protein resident in membrane rafts and plasmodesmata, impairs potato virus X movement. Plant Cell. 2009;21:1541-1555.

239. Ray S, Yumak H, Domashevskiy A, et al. Tobacco etch virus mRNA preferentially binds wheat germ eukaryotic initiation factor (eIF) 4G rather than eIFiso4G. J Biol Chem. 2006;281:35826-35234.

240. Reagan BC, Ganusova EE, Fernandez JC, et al. RNA on the move: the plasmodesmata perspective. Plant Sci. 2018;275:1-10.

241. Revers F, Antonio Garcia J. Molecular biology of potyviruses. Adv Virus Res. 2015;92:101-199.

242. Richard MMS, Knip M, Schachtschabel J, et al. Perturbation of nuclear-cytosolic shuttling of Rx1 compromises extreme resistance and translational arrest of potato virus X transcripts. Plant J. 2021;106:468-479.

243. Roberts R, Mayberry LK, Browning KS, et al. The triticum mosaic virus 5′ leader binds to both eIF4G and elFiso4G for translation. PLoS One. 2017;12:e0169602.

244. Roberts R, Zhang JC, Mayberry LK, et al. A unique 5′ translation element discovered in triticum mosaic virus. J Virol. 2015;89:12427-12440.

245. Roossinck MJ. The good viruses: viral mutualistic symbioses. Nat Rev Microbiol. 2011;9:99-108.

246. Roossinck MJ. Plant virus metagenomics: biodiversity and ecology. Annu Rev Genet. 2012;46:359-369.

247. Roossinck MJ, Martin DP, Roumagnac P. Plant virus metagenomics: advances in virus discovery. Phytopathology. 2015;105:716-727.

248. Rosas-Diaz T, Zhang D, Fan PF, et al. A virus-targeted plant receptor-like kinase promotes cell-to-cell spread of RNAi. Proc Natl Acad Sci U S A. 2018;115:1388-13893.

249. Ruggenthaler P, Fichtenbauer D, Krasensky J, et al. Microtubule-associated protein atmpb2c plays a role in organization of cortical microtubules, stomata patterning, and tobamovirus infectivity. Plant Physiol. 2009;149:1354-1365.

250. Sainsbury F, Lavoie P-O, D′Aoust M-A, et al. Expression of multiple proteins using full-length and deleted versions of cowpea mosaic virus RNA-2. Plant Biotechnol J. 2008;6:82-92.

251. Sakamoto T, Deguchi M, Brustolini OJB, et al. The tomato RLK superfamily: phylogeny and functional predictions about the role of the LRRII-RLK subfamily in antiviral defense. BMC Plant Biol. 2012;12:229.

252. Sakurai Y, Baeg K, Lam AYW, et al. Cell-free reconstitution reveals the molecular mechanisms for the initiation of secondary siRNA biogenesis in plants. Proc Nalt Acad Sci U S A. 2021;118:e2102889118.

253. Sano T. Progress in 50 years of viroid research-Molecular structure, pathogenicity, and host adaptation. Proc Jpn Acad Ser B Phys Biol Sci. 2021;97:371-401.

254. Scorrano L, De Matteis MA, Emr S, et al. Coming together to define membrane contact sites. Nat Commun. 2019;10:1287.

255. Semashko MA, Gonzalez I, Shaw J, et al. The extreme N-terminal domain of a hordeivirus TGB1 movement protein mediates its localization to the nucleolus and interaction with fibrillarin. Biochimie. 2012;94:1180-1188.

256. Semashko MA, Rakitina DV, Gonzalez I, et al. Movement protein of hordeivirus interacts in vitro and in vivo with coilin, a major structural protein of Cajal bodies. Dokl Biochem Biophys. 2012;442:57-60.

257. Senthil-Kumar M, Mysore KS. New dimensions for VIGS in plant functional genomics. Trends Plant Sci. 2011;16:656-665.

258. Shen J, Chen X, Chen J, Sun L. A phloem-limited fijivirus induces the formation of neoplastic phloem tissues that house virus multiplication in the host plant. Sci Rep. 2016;6:29848.

259. Shen QT, Hu T, Bao M, et al. Tobacco RING E3 ligase NtRFP1 mediates ubiquitination and proteasomal degradation of a geminivirus-encoded βC1. Mol Plant. 2016;9:911-925.

260. Shen W, Hanley-Bowdoin L. SnRK1: a versatile plant protein kinase that limits geminivirus infection. Curr Opin Virol. 2021;47:18-24.

261. Shi M, Lin XD, Chen X, et al. The evolutionary history of vertebrate RNA viruses. Nature. 2018;556:197-202.

262. Shukla A, Hoffmann G, Kushwaha NK, et al. Salicylic acid and the viral virulence factor 2b regulate the divergent roles of autophagy during cucumber mosaic virus infection. Autophagy. 2022;18(6):1450-1462. doi:10.1080/15548627.2021.1987674

263. Shukla S, Wen AM, Ayat NR, et al. Biodistribution and clearance of a filamentous plant virus in healthy and tumor-bearing mice. Nanomedicine. 2014;9:221-236.

264. Silva-Martins G, Bolaji A, Moffett P. What does it take to be antiviral? An Argonaute-centered perspective on plant antiviral defense. J Exp Bot. 2020;71:6197-6210.

265. Simon AE, Miller WA. 3′ Cap-independent translation enhancers of plant viruses. Annu Rev Microbiol. 2013;67:21-42.

266. Simon AE, Roossinck MJ, Havelda Z. Plant virus satellite and defective interfering RNAs: new paradigms for a new century. Annu Rev Phytopathol. 2004;42:415-437.

267. Sorokin, II, Vassilenko KS, Terenin IM, et al. Non-canonical translation initiation mechanisms employed by eukaryotic viral mRNAs. Biochemistry (Mosc). 2021;86:1060-1094.

268. Steele JFC, Peyret H, Saunders K, et al. Synthetic plant virology for nanobiotechnology and nanomedicine. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2017;9:e1447.

269. Steinmetz NF, Lin T, Lomonossoff GP, Johnson JE. Structure-based engineering of an icosahedral virus for nanomedicine and

nanotechnology. Curr Top Microbiol Immunol. 2009;327:23-58.

270. Stewart LR, Ding B, Falk BW. Viroids and phloem-limited viruses: unique molecular probes of phloem biology. In: van Bel AJE, eds. *Phloem*: *Molecular Cell Biology*, *Systemic Communication*, *Biotic Interactions*. John Wiley & Sons, Inc.; 2013:271-292.

271. Storms MMH, Kormelink R, Peters D, et al. The nonstructural NSm protein of tomato spotted wilt virus induces tubular structures in plant and insect cells. Virology. 1995;214:485-493.

272. Sun R, Han J, Zheng L, Qu F. The AC2 protein of a bipartite geminivirus stimulates the transcription of the BV1 gene through abscisic acid responsive promoter elements. Viruses. 2020;1:1403.

273. Sun YD, Folimonova SY. Location matters: from changing a presumption about the Citrus tristeza virus tissue tropism to understanding the stem pitting disease. New Phytol. 2022;233:631-638.

274. Suttle CA. Marine viruses—major players in the global ecosystem. Nat Rev Microbiol. 2007;5:801-812.

275. Sztuba-Solinska J, Fanning SW, Horn JR, et al. Mutations in the coat protein-binding cis-acting RNA motifs debilitate RNA recombination of Brome mosaic virus. Virus Res. 2012;170:138-149.

276. Taliansky ME, Robinson DJ. Molecular biology of umbraviruses: phantom warriors. J Gen Virol. 2003;84:1951-1960.

277. Tan ZY, Wada Y, Chen JS, Ohshima K. Inter- and intralineage recombinants are common in natural populations of turnip mosaic virus. J Gen Virol. 2004;85:2683-2696.

278. Terret-Welter Z, Bonnet G, Moury B, et al. Analysis of tomato spotted wilt virus RNA-dependent RNA polymerase adaptative evolution and constrained domains using homology protein structure modelling. J Gen Virol. 2020;101:334-346.

279. Thuenemann EC, Byrne MJ, Peyret H, et al. A replicating viral vector greatly enhances accumulation of helical virus-like particles in plants. Viruses. 2021;13:885.

280. Tilsner J, Linnik O, Louveaux M, et al. Replication and trafficking of a plant virus are coupled at the entrances of plasmodesmata. J Cell Biol. 2013;201:981-995.

281. Tilsner J, Linnik O, Wright KM, et al. The TGB1 movement protein of potato virus X reorganizes actin and endomembranes into the X-body, a viral replication factory. Plant Physiol. 2012;158:1359-1370.

282. Tilsner J, Oparka KJ. Missing links? The connection between replication and movement of plant RNA viruses. Curr Opin Virol. 2012;2:705-711.

283. Torti S, Schlesier R, Thümmler A, et al. Transient reprogramming of crop plants for agronomic performance. Nat Plants. 2021;7:159-171.

284. Trebicki P. Climate change and plant virus epidemiology. Virus Res. 2020;286:198059.

285. Tromas N, Zwart MP, Poulain M, et al. Estimation of the in vivo recombination rate for a plant RNA virus. J Gen Virol. 2014;95:724-732.

286. Truniger V, Miras M, Aranda MA. Structural and functional diversity of plant virus 3'-cap-independent translation enhancers (3′-CITEs). Front Plant Sci. 2017;8:2047.

287. Uchiyama A, Shimada-Beltran H, Levy A, et al. The Arabidopsis synaptotagmin SYTA regulates the cell-to-cell movement of diverse plant viruses. Front Plant Sci. 2014;5:584.

288. Valli A, Gallo A, Calvo M, et al. A novel role of the potyviral helper component proteinase contributes to enhance the yield of viral particles. J Virol. 2014;88:9808-9818.

289. Valli AA, Gallo A, Rodamilans B, et al. The HCPro from the potyviridae family: an enviable multitasking helper component that every virus would like to have. Mol Plant Pathol. 2018;19:744-763.

290. Velasco L, Padilla CV. High-throughput sequencing of small RNAs for the sanitary certification of viruses in grapevine. Front Plant Sci. 2021;12:682879.

291. Veluthambi K, Sunitha S. Targets and mechanisms of geminivirus silencing suppressor protein AC2. Front Microbiol. 2021;12:645419.

292. Venkataraman S, Badar U, Shoeb E, et al. An inside look into biological miniatures: molecular mechanisms of viroids. Int J Mol Sci. 2021;22:2795.

293. Venkataraman S, Hefferon K. Application of plant viruses in biotechnology, medicine, and human health. Viruses. 2021;13:1697.

294. Verchot J, Driskel BA, Zhu Y, et al. Evidence that soilborne wheat mosaic virus moves long distance through the xylem in wheat. Protoplasma. 2001;218:57-66.

295. Verchot J, Herath V, Urrutia CD, et al. Development of a reverse genetic system for studying rose rosette virus in whole plants. Mol Plant Microbe Interact. 2020;33:1209-1221.

296. Verchot J, Pajerowska-Mukhtar KM. UPR signaling at the nexus of plant viral, bacterial, and fungal defenses. Curr Opin Virol. 2021;47:9-17.

297. Verchot-Lubicz J, Torrance L, Solovyev AG, et al. Varied movement strategies employed by triple gene block-encoding viruses. Mol Plant Microbe Interact. 2010;23:1231-1247.

298. Vijayapalani P, Maeshima M, Nagasaki-Takekuchi N, et al. Interaction of the trans-frame potyvirus protein P3N-PIPO with host protein PCaP1 facilitates potyvirus movement. PLoS Pathog. 2012;8:e1002639.

299. Wan J, Basu K, Mui J, et al. Ultrastructural characterization of turnip mosaic virus-induced cellular rearrangements reveals membrane-bound viral particles accumulating in vacuoles. J Virol. 2015;89:12441-12456.

300. Wan J, Cabanillas DG, Zheng H, et al. Turnip mosaic virus moves systemically through both phloem and xylem as membrane-associated complexes. Plant Physiol. 2015;67:1374-1388.

301. Wang AM. Cell-to-cell movement of plant viruses via plasmodesmata: a current perspective on potyviruses. Curr Opin Virol. 2021;48:10-16.

302. Wang JZ, Hu MJ, Wang J, et al. Reconstitution and structure of a plant NLR resistosome conferring immunity. Science. 2019;364:eaav5870.

303. Wang Y, Ding B. Viroids: small probes for exploring the vast universe of RNA trafficking in plants. J Integr Plant Biol. 2010;52:28-39.

304. Wang Y, Qiao R, Wei CH, et al. Rice dwarf virus small RNA profiles in rice and leafhopper reveal distinct patterns in cross-kingdom hosts. Viruses. 2019;11:847.

305. Wang YJ, Gong Q, Wu YY, et al. A calmodulin-binding transcription factor links calcium signaling to antiviral RNAi defense in plants. Cell Host Microbe. 2021;29:1393-1406.e7.

306. Wang X, Olmedo-Velarde A, Larrea-Sarmiento A, et al. Genome characterization of fig umbra-like virus. Virus Genes. 2021;57:566-570.

307. Weathers L. A vein-yellowing disease of citrus caused by a graft-transmissible virus. Plant Dis Reptr. 1957;41:741-742.

308. Wei T, Li Y. Rice reoviruses in insect vectors. Annu Rev Phytopathol. 2016;54:99-120.

309. Wei T, Wang A. Biogenesis of cytoplasmic membranous vesicles for plant potyvirus replication occurs at endoplasmic reticulum exit sites in a COPI- and COPII-dependent manner. J Virol. 2008;82:12252-12264.

310. Wei T, Zhang C, Hou X, et al. The SNARE Protein Syp71 is essential for turnip mosaic virus infection by mediating fusion of virus-induced vesicles with chloroplasts. PLoS Pathog. 2013;9:e1003378.

311. Wei TY, Zhang CW, Hong JA, et al. Formation of complexes at plasmodesmata for potyvirus intercellular movement is mediated by the viral protein P3N-PIPO. PLoS Pathog. 2010;6:e1000962.

312. Wen AM, Steinmetz NF. Design of virus-based nanomaterials for medicine, biotechnology, and energy. Chem Soc Rev. 2016;45:4074-4126.

313. Westwood JH, McCann L, Naish M, et al. A viral RNA silencing suppressor interferes with abscisic acid-mediated signalling and induces drought tolerance in Arabidopsis thaliana. Mol Plant Pathol. 2013;14:158-170.

314. Wright KM, Cowan GH, Lukhovitskaya NI, et al. The N-terminal domain of PMTV TGB1 movement protein is required for nucleolar localization, microtubule association, and long-distance movement. Mol Plant Microbe Interact. 2010;23:1486-1497.

315. Wu D, Qi T, Li W-X, et al. Viral effector protein manipulates host hormone signaling to attract insect vectors. Cell Res. 2017;27:402-415.

316. Wu G, Cui X, Chen H, et al. Dynamin-like proteins of endocytosis in plants are coopted by potyviruses to enhance virus infection. J Virol. 2018;92:e01320-18.

317. Wu JG, Yang RX, Yang ZR, et al. ROS accumulation and antiviral defence control by microRNA528 in rice. Nat Plants. 2017;3:16203.

318. Wu JG, Yang ZR, Wang Y, et al. Viral-inducible Argonaute18 confers broad-spectrum virus resistance in rice by sequestering a host microRNA. Elife. 2015;4:e05733.

319. Wu MS, Wei H, Tan H, et al. Plant DNA polymerases α and δ mediate replication of geminiviruses. Nat Commun. 2021;12:2780.

320. Wu QF, Ding SW, Zhang YJ, et al. Identification of viruses and viroids by next-generation sequencing and homology-dependent and homology-independent algorithms. Annu Rev Phytopathol. 2015;53:425-444.

321. Wu QF, Luo YJ, Lu R, et al. Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs. Proc Natl Acad Sci U S A. 2010;107:1606-1611.

322. Wu XY, Cheng XF. Intercellular movement of plantRNAviruses: Targeting replication complexes to the plasmodesma for both accuracy and efficiency. Traffic. 2020;21: 725-736.

323. Wu XY, Valli A, Garcia JA, et al. The tug-of-war between plants and viruses: great progress and many remaining questions. Viruses. 2019;11:203.

324. Wu Y, Cao S, Alam MNA, et al. Fluorescent nanodiamonds encapsulated by cowpea chlorotic mottle virus (CCMV) proteins for intracellular 3D-trajectory analysis. J Mater Chem B. 2021;9:5621-5627.

325. Xiao YH, Rouzine IM, Bianco S, et al. RNA recombination enhances adaptability and is required for virus spread and virulence. Cell Host Microbe. 2016;19:493-503.

326. Xie L, Lv MF, Zhang HM, et al. Tumours induced by a plant virus are derived from vascular tissue and have multiple intercellular gateways that facilitate virus movement. J Exp Bot. 2014;65:4873-4886.

327. Xu K, Nagy PD. Sterol binding by the tombusviral replication proteins is essential for replication in yeast and plants. J Virol. 2017;91:e01984-16.

328. Xu P, Chen F, Mannas JP, et al. Virus infection improves drought tolerance. New Phytol. 2008;180:911-921.

329. Yan C, Fan M, Yang M, et al. Injury activates Ca2+/calmodulin-dependent phosphorylation of JAV1-JAZ8-WRKY51 complex for jasmonate biosynthesis. Mol Cell. 2018;70:136-149.e7.

330. Yang XL, Li YZ, Wang AM. Research advances in potyviruses: from the laboratory bench to the field. Annu Rev Phytopathol. 2021;59:1-29.

331. Yang Y, Yang K, Wang J, et al. Fabrication and characterization of CdS nanowires templated in tobacco mosaic virus with improved photocatalytic ability. Appl Microbiol Biotechnol. 2021;105:8255-8264.

332. Yang Z, Li Y. Dissection of RNAi-based antiviral immunity in plants. Curr Opin Virol. 2018;32:88-99.

333. Yang ZR, Huang Y, Yang JL, et al. Jasmonate signaling enhances RNA silencing and antiviral defense in rice. Cell Host Microbe. 2020;28:89-103.e8.

334. Ye J, Zhang L, Zhang X, et al. Plant defense networks against insect-borne pathogens. Trends Plant Sci. 2021;26:272-287.

335. Yu M, Bi X, Huang Y, et al. Chimeric tobamoviruses with coat protein exchanges modulate symptom expression and defence responses in *Nicotiana tabacum*. Front Microbiol. 2020;11:587005.

336. Yu XZ, Jia DS, Wang Z, et al. A plant reovirus hijacks endoplasmic reticulum-associated degradation machinery to promote efficient viral transmission by its planthopper vector under high temperature conditions. PLoS Pathog. 2021;17:e1009347.

337. Yuan C, Lazarowitz SG, Citovsky V. Identification of a functional plasmodesmal localization signal in a plant viral cell-to-cell-movement protein. mBio. 2016;7:e02052-15.

338. Yuan X, Shi K, Meskauskas A, et al. The 3′ end of turnip crinkle virus contains a highly interactive structure including a translational enhancer that is disrupted by binding to the RNA-dependent RNA polymerase. RNA. 2009;15:1849-1864.

339. Zarreen F, Chakraborty S. Epigenetic regulation of geminivirus pathogenesis: a case of relentless recalibration of defence responses in plants. J Exp Bot. 2020;71:6890-6906.

340. Zeenko V, Gallie DR. Cap-independent translation of tobacco etch virus is conferred by an RNA pseudoknot in the 5′-leader. J Biol Chem. 2005;280:26813-26824.

341. Zhang C, Ding ZM, Wu KC, et al. Suppression of jasmonic acid-mediated defense by viral-inducible microRNA319 facilitates virus infection in rice. Mol Plant. 2016;9: 1302-1314.

342. Zhang C, Wei Y, Xu L, et al. A bunyavirus-inducible ubiquitin ligase targets RNA Polymerase IV for degradation during viral pathogenesis in rice. Mol Plant. 2020;13:836-850.

343. Zhang C, Wu Z, Li Y, et al. Biogenesis, function, and applications of virus-derived small rnas in plants. Front Microbiol. 2015;6:1237.

344. Zhang T, Luan J-B, Qi J-F, et al. Begomovirus-whitefly mutualism is achieved through repression of plant defences by a virus pathogenicity factor. Mol Ecol. 2012;21:1294-1304.

345. Zhang X, Lai TF, Zhang PC, et al. Mini review: revisiting mobile RNA silencing in plants. Plant Sci. 2019;278:113-117.

346. Zhang XX, Yin YY, Su YH, et al. eIF4A, a target of siRNA derived from rice stripe virus, negatively regulates antiviral autophagy by interacting with ATG5 in *Nicotiana benthamiana*. PLoS Pathog. 2021;17:e1009963.

347. Zhao P, Liu Q, Miller WA, et al. Eukaryotic translation initiation factor 4G (eIF4G) coordinates interactions with eIF4A, eIF4B, and eIF4E in binding and translation of the barley yellow dwarf virus 3' cap-independent translation element (BTE). J Biol Chem. 2017;292:5921-5931.

348. Zhao P, Zhang X, Gong Y, et al. Red-light is an environmental effector for mutualism between begomovirus and its vector whitefly. PLoS Pathog. 2021;17:e1008770.

349. Zhao SS, Li Y. Current understanding of the interplays between host hormones and plant viral infections. PLoS Pathog. 2021;17:e1009242.

350. Zhao W, Xu ZT, Zhang XM, et al. Genomic variations in the 3′-termini of rice stripe virus in the rotation between vector insect and host plant. New Phytol. 2018;219:1085-1096.

351. Zhong XT, Wang ZQ, Xiao RY, et al. Mimic phosphorylation of a βC1 protein encoded by TYLCCNB impairs its functions as a viral suppressor of RNA silencing and a symptom determinant. J Virol. 2017;91(16):e00300-17. https://doi.org/10.1128/JVI.00300-17

352. Zhou T, Dong L, Jiang T, et al. Silencing specific genes in plants using virus-induced gene silencing (VIGS) vectors. Methods Mol Biol. 2022;2400:149-161.

353. Zhou XP. Advances in understanding begomovirus satellites. Annu Rev Phytopathol. 2013;51:357-381.

354. Zhu M, Jiang L, Bai BH, et al. The intracellular immune receptor Sw-5b confers broad-spectrum resistance to tospoviruses through recognition of a conserved 21-amino acid viral effector epitope. Plant Cell. 2017;29:2214-2232.

355. Zorzatto C, Machado JPB, Lopes KVG, et al. NIK1-mediated translation suppression functions as a plant antiviral immunity mechanism. Nature. 2015;520:679-682.

356. Zvereva AS, Golyaev V, Turco S, et al. Viral protein suppresses oxidative burst and salicylic acid-dependent autophagy and facilitates bacterial growth on virus-infected plants. New Phytol. 2016;211:1020-1034.

CHAPTER 19

Insect Viruses

Paul D. Friesen

Impact of Insect Viruses

Discovery and classification of insect viruses

Biology, virus interactions, and virus evolution

Expression vectors for foreign genes

Gene transduction, delivery, and gene therapy

Biopesticide viruses for insect control

Description of insect virus families

Baculoviridae

Polydnaviridae (*Polydnaviriformidae*)

Ascoviridae

Nudiviruses

Nodaviridae

Tetraviridae

Dicistroviridae

Perspectives

Impact of Insect Viruses

Insects comprise well over half of the existing animals on earth. They include an estimated 5.5 million species and represent some of the most diverse and successful animals on the planet.[302] Accordingly, the RNA and DNA viruses infecting insects are also highly diverse, the likely result of 350 million years of coevolution with their hosts. Because insect viruses inflict disease and mortality in their hosts and multiple virus species can cross over into vertebrates and plants, they have long been of concern for human health and agriculture. Hence, much of the early research on these diverse viruses was dedicated to understanding the impact of pathogenic insect viruses on human health and agriculture, insect control included. As molecular studies of insect viruses advanced, it was recognized that these obligate parasites also provided valuable tools for investigating fundamental questions in cellular and molecular biology. Such studies have led to advances and direct applications in many fields, including biochemistry, cell biology, biotechnology, gene therapy, vaccine production, epidemiology, and insect management.

Discovery and Classification of Insect Viruses

Insects are susceptible to highly diverse families of DNA and RNA viruses (Table 19.1). While some insect virus families contain members that exclusively infect arthropod hosts, other virus families contain members that infect both invertebrate and vertebrate hosts. Indeed, many insect virus families have the capacity to multiply in vertebrate hosts upon introduction by an insect vector, causing numerous well-known and prevalent diseases. Some insect virus families have closely related counterparts that exclusively infect vertebrate hosts. For example, the invertebrate subfamily *Entomopoxvirinae* and the vertebrate subfamily *Chordopoxvirinae* are both part of the larger *Poxviridae* family. Interestingly, insect viruses often have novel properties that are common amongst insect-specific virus families and function to facilitate invertebrate infection. A striking example is the existence of an occluded virus particle for virus transmission between insect hosts. This morphological feature is conserved among diverse virus families, including the baculoviruses (*Baculoviridae*), entomopoxviruses (*Poxviridae*), cypoviruses (*Reoviridae*) and possibly the nudiviruses (*Nudiviridae*).

TABLE 19.1 Virus families infecting the insects

Characteristics	Virus Family	Genus	Representative Member
dsDNA, enveloped	Baculoviridae	*Nucleopolyhedrovirus*	*Autographa californica* multiple NPV (Ac*M*NPV)
		Granulovirus (GV)	*Cydia pomonella* GV (CpGV)
	Polydnaviriformidae	*Ichnovirus*	*Campoletis sonorensis* ichnovirus (CsIV)
		Bracovirus	*Cotesia melanoscela* bracovirus (CcBV)
	Ascoviridae	*Ascovirus*	*Spodoptera frugiperda* ascovirus 1a (SfAV-1a)
	Nudiviruses	*Alphanudivirus*	*Oryctes rhinoceros* virus
		Betanudivirus	*Heliothus zea* virus 1 (HzV-1)
	Poxviridae	*Alphaentomopoxvirus*	*Melolontha melolontha* entomopoxvirus
		Betaentomopoxvirus	*Amsacta moorei* entomopoxvirus "L"
		Gammaentomopoxvirus	*Chironomus luridus* entomopoxvirus
dsDNA, nonenveloped	Iridoviridae	*Iridovirus*	Invertebrate iridescent virus 6 (IIV-6)
		Chloriridovirus	Invertebrate iridescent virus 3 (IIV-3)
ssDNA, nonenveloped	Parvoviridae	*Densovirus*	*Junonia coenia* densovirus
		Pefudensovirus	*Periplaneta fuliginosa* densovirus
		Brevidensovirus	*Aedes aegypti* densovirus
		Iteravirus	*Bombyx mori* densovirus
dsRNA, nonenveloped	Reoviridae	*Orbivirus*	Bluetongue virus
		Cypovirus	Cypovirus 1 (ie, BmCPV)
		Coltivirus	Colorado tick fever virus
		Seadornavirus	Banna virus
		Idnoreovirus	Idnoreovirus 1
		Fijivirus	Fiji disease virus
		Phytoreovirus	Wound tumor virus
		Oryzavirus	Rice ragged stunt virus
	Birnaviridae	*Entomobirnavirus*	*Drosophila* X virus
ssRNA, enveloped	Togaviridae	*Alphavirus*	Sindbis virus
	Flaviviridae	*Flavivirus*	Yellow fever virus
	Rhabdoviridae	*Vesiculovirus*	Vesicular stomatitis Indiana virus
	Bunyaviridae	*Orthobunyavirus*	Bunyamwera virus
		Phlebovirus	Rift Valley fever virus
		Nairovirus	Dugbe virus
Retrotransposons	Metaviridae	*Errantivirus*	*Drosophila* gypsy virus
		Semotivirus	*Bombyx mori* Pao virus
ssRNA, nonenveloped	Nodaviridae	*Alphanodavirus*	Nodamura virus, Flock House virus
	Tetraviridae	*Betatetravirus*	*Nudaurelia capensis* β virus
		Omegatetravirus	*Nudaurelia capensis* ω virus
	Dicistroviridae	*Cripavirus*	Cricket paralysis virus
			Drosophila C virus
	Iflavirus	*Iflavirus*	Infectious flacherie virus (IFV)
			Deformed wing virus
			Slow bee paralysis virus

Many insect viruses were originally discovered by their associated disease and subsequently classified by using standard methods for virus isolation, biochemical characterization, and genetic analyses. Recently, high throughput, next-generation sequencing of invertebrate genomes has led to the discovery of an enormous number of hitherto unknown insect viruses.[27] On the basis of sequence similarities, some of these new viruslike entities are members of previously known virus families infecting both invertebrates and vertebrates while others represent newly discovered families.[291,351] In surveys of >200 insect species, the RNA viruses predominate; the scale is enormous. These putative viruses were identified by their gene sequences that share distant similarity to known RNA-dependent RNA polymerases, which represent the most conserved of the RNA virus genes. Fewer DNA viruses related to known DNA virus families or having similarity to known DNA-dependent DNA polymerases have been identified in these surveys. These RNA and DNA elements are putative viruses as they have only been identified by metagenomic analyses.[291,351] While some of these putative viruses could represent chronic, latent, or asymptomatic infections, others could be derived from microorganisms associated with the insect or the insect's meal acquired prior to analysis. Interestingly, another source of virus-related sequences in insects are those likely derived by reverse-transcription (the activity of endogenous retro-elements) of insect RNA virus genomes that integrate within the host genome or are stably maintained.[109] These full-length or partial genomic sequences can function in an antiviral response that uses the well-characterized RNA interference (RNAi) machinery of insects.[258] Much work remains to verify the infectious nature of viruslike metagenomic sequences and to subsequently evaluate their potential applications.

This chapter describes those characterized viruses that multiply primarily or exclusively in insects: baculoviruses, polydnaviruses (within *Polydnaviriformidae*), ascoviruses, nudiviruses, nodaviruses, tetraviruses, and the dicistroviruses. Viruses such as the arboviruses in which insects serve as host vectors during transmission to vertebrates are described in other chapters. Insect virus families such as the

entomopoxviruses, densoviruses, iridescent viruses, cypoviruses, and iflaviruses have also been described elsewhere.[3,117,155,160,355]

Biology, Virus Interactions, and Virus Evolution

Biochemical and genetic studies on insect virus multiplication and host pathogenesis have contributed enormously to our understanding of molecular and cellular biology, virus structure and assembly, virus-host interactions, and disease states. These advances are due in a large part to the diversity of insect viruses and the availability and convenience of cell cultures or live insects in which to propagate viruses. Such systems, when combined with the ease with which insect viruses can be engineered or genetically manipulated, provide remarkably high yields of virus particles or virus-encoded macromolecules for studies of gene structure and biochemical function. There are also distinct experimental advantages to the use of many insect hosts, including those with sequenced and annotated genomes. Compared to vertebrates, insects exhibit limited genetic redundancy, a property that has simplified studies on host-virus interaction pathways. Finally, there exists a striking conservation of genes and genetic pathways between insects and mammals. Thus, discoveries from studies of insect viruses and their hosts have led to important biological insights for all animals, humans included.

Insect viruses have provided fundamental information on cell biology and virus-host interactions. For example, the insect cypoviruses of the family *Reoviridae* were the first to reveal the critical nature of the 7-methyl GpppN cap on eukaryotic mRNAs.[100] Novel internal ribosomal entry sites (IRES) and new mechanisms for initiation of protein synthesis[143] have been revealed by studies of cricket paralysis virus of the family *Dicistroviridae*. The nodaviruses provided the first example of an animal (insect) virus-encoded suppressor of host RNA silencing (RNAi)[192]; RNAi suppressors have now been identified in multiple animal viruses. Studies on the nodaviruses have also revealed new macromolecular structures responsible for RNA-dependent RNA polymerase-mediated RNA replication in membrane-bound vesicles, which may be common among multiple positive-sense RNA virus families.[325,326] The discovery of baculovirus-encoded inhibitor of apoptosis (IAP) genes led to the identification of IAP homologs in animals and subsequently established the critical role of IAPs in regulating apoptosis in normal development, immune function, and human diseases, including cancer and neurodegeneration.[59,231,298] The baculoviruses encode other novel suppressors of apoptosis that have provided important insight into the role of apoptosis as an antiviral defense in insects and mammals.[58] Lastly, the polydnaviruses have disclosed a remarkable advantageous relationship between virus and host that involves virus-mediated immune suppression that facilitates virus and host survival.[304,305] Thus, insect viruses have collectively provided key understanding in numerous aspects of cell biology, pathogenesis, and host-virus interactions.

Insect viruses have also provided unique insight into virus evolution. Due to the close association of insects with plants and other animals, natural exchange and mixing of viruses is accelerated. Insect viruses within the families *Poxviridae*, *Rhabdoviridae*, *Reoviridae*, *Picornaviridae*, and *Parvoviridae* have vertebrate counterparts, leading to speculation that viruses of higher animals arose from insect virus ancestors. Certain insect viruses have coevolved with unrelated insect virus families. For example, it is likely that the DNA baculoviruses and the retroviruslike errantiviruses, both of which coexist or multiply in lepidopteran hosts, have exchanged envelope fusion protein genes as suggested by the striking the sequence similarity of these proteins.[252,275] Thus, despite their dissimilar replication strategies, these viruses may have coevolved. The striking conservation of specific genes among diverse insect virus families, including baculoviruses, entomopoxviruses, orthomyxoviruses, ascoviruses, and iridoviruses as well as the recently discovered relationship between viruses of *Polydnaviridae* (*Polydnaviriformidae*) and *Nudiviridae* is consistent with coevolution of invertebrate viruses. It is noteworthy that certain DNA viruses of insects also readily accommodate host-derived transposons and retroid elements, which are especially abundant in arthropods. Stable retention of such host-derived mobile genetic elements facilitates virus evolution and likely contributes to the exchange of genes between virus and its host organism.[105,107]

Expression Vectors for Foreign Genes

Among eukaryotic viruses, the insect viruses are among the best known for their use as vectors for foreign gene expression because of their productivity, efficiency, and utility. The most widely used vectors are those derived from the helper-independent baculoviruses.[44,50,157,166,173,327] This widespread biotechnological application is the direct result of intense molecular studies on baculovirus genetics, biochemistry, and multiplication strategies; it represents a striking example of the utility of insect viruses for basic and applied research. The remarkable strength of very late baculoviral promoters, the high level of virus multiplication, the extraordinary capacity for protein synthesis in cultured insect cells, and the ability to maintain large foreign DNA inserts are the principal factors that contribute to the efficiency and popularity of baculoviruses as eukaryotic expression vectors. The most used baculovirus expression vector system is that derived from *Autographa californica* nucleopolyhedrovirus (Ac*M*NPV), a nuclear-replicating DNA virus that can generate virus-specific macromolecules that comprise well over one-third of the mass of the infected cell. Thousands of different heterologous proteins have been expressed and subsequently studied by using the Ac*M*NPV vector system since its original development in the early 1980s by the independent laboratories of Max Summers and Lois Miller.[255,295]

Baculovirus vectors have been improved significantly for speed of recombinant virus generation, purification, and production of a large variety protein types, including cytoplasmic, nuclear, secreted, and membrane-associated polypeptides.[259] Methods for recombinant vector production are now efficient and routine. Strategies for insertion and simultaneous expression of multiple genes or for temporally regulated, promoter-specific expression are available. Since posttranslational modifications and biological activity of the resulting protein products often mimic or parallel that of mammalian cells, these vectors have provided abundant material for applications in diagnostics, therapeutics, and vaccine development, as well as general studies on protein structure and function. Like other expression systems, however, baculovirus vectors have advantages and disadvantages that depend on the type of protein to be produced.[157,259,327] Insect cell lines have also been improved by adaptation to serum-free media and specific engineering for glycosylation of protein products that mimics that of mammalian cells.[157] Live insect larvae have also been used as effective and convenient large-scale bioreactors for the production of high levels of many important proteins. For example, *Bombyx mori* nucleopolyhedrovirus (BmNPV) has been used as an efficient vector for protein production in silkworms.[166] For FDA-approved antigen preparation, baculoviruses have also been used to express heterologous proteins and peptides.[173,327]

The promoters for certain immediate early baculovirus genes are highly active in the absence of virus proteins.[175,178,266,318] As a result, several of these baculovirus promoters, including those for the IE1 and IE2 genes, have been used extensively for high-level gene expression in diverse lepidopteran and dipteran insects by transient transfections or the generation of stable cell lines.[137] This approach allows long-term, high-level expression in the absence of virus vectors.

It is noteworthy that other insect viruses, including the entomopoxviruses,[256] densoviruses, and alphaviruses, have also been exploited as expression vectors. Likewise, flock house virus of the RNA virus family *Nodaviridae* has been used to express foreign epitopes on the surface of its icosahedral virion particle.[333] FHV has also been used as a dsRNA delivery vector for RNAi-mediated gene silencing.[314]

Gene Transduction, Delivery, and Gene Therapy

As a result of recent advances, the baculovirus Ac*M*NPV has been used effectively as a gene transfer vector for a surprising variety of vertebrate cells, including those from mammals.[50,239,281] Although baculoviruses can enter mammalian cells, they do not multiply nor cause widespread cell death. Thus, when inserted into the baculovirus genome under control of appropriate mammalian transcriptional signals (promoters and enhancers), foreign genes can be delivered and expressed after inoculation of mammalian cell lines in a process referred to as "transduction." Expression is transient and levels vary depending on the protein, the mammalian promoter used, and the target cells. The mechanism of virus entry is poorly understood and is likely different depending on cell or tissue type. The Ac*M*NPV envelope fusion protein GP64 (see below) is required (see below).

Because of its lack of multiplication in mammalian cells, its low cytotoxicity, the capacity for foreign gene expression, and its high transduction efficiency, Ac*M*NPV has also been evaluated as a gene therapy vector.[50,239,281,344] Applications have included its use in cancer therapy, regenerative medicine, vaccine vectors, and gene product ablation by RNA interference. In the case of baculovirus-mediated cancer therapeutics, tested approaches include virus-mediated expression of tumor-specific antigens for anticancer vaccines, introduction of functional tumor suppressors (eg, P53) or proapoptotic genes, and the delivery and expression of suicide genes that confer tumor-specific susceptibility to select toxins or chemotherapeutics drugs. It is expected that these novel therapeutic approaches that exploit insect viruses will receive more attention in the future. Indeed, Ac*M*NPV vectors have already found use in the production of adenovirus-associated virus gene therapy reagents.[161,259,280]

Biopesticide Viruses for Insect Control

While comprising over 80% of the existing animal species on earth, many insects present serious biomedical adversity for humans and cause significant agricultural and forest damage on a worldwide scale. Thus, environmentally safe means of controlling insect populations is a major objective. Insect populations are naturally held in balance by predation, parasitism, and infection by microbial pathogens. Of these diverse factors, viruses have numerous practical advantages for deployment as insect control agents and already represent viable components in current integrated pest management strategies.[45,183,275]

In nature, viruses cause widespread epizootics and morbidity in insects, especially within dense populations. Spontaneous epizootics by viruses have occurred frequently in agricultural and forest settings.[275] Nonetheless, dependence upon epizootics for insect control has been impractical due to the unacceptable levels of crop damage that occur before the pest population declines. Thus, much effort has been invested in the development of viral biopesticides for agricultural purposes. Several viruses have already been used as effective, safe, and highly specific biocontrol agents that have the added benefit of reducing dependence on broad-range, toxic chemical insecticides. Of these viruses, the DNA baculoviruses have received the most attention due to their relative safety in the environment, their insect-specific virulence, the capacity for large-scale production, and the availability of effective formulations for field application.[308]

As one of the most successful applications of viral pesticides, the baculovirus *Anticarsia gemmatalis* multiple nucleopolyhedrovirus (AgMNPV) has been used widely to control the soybean looper on a large scale, especially in Brazil.[221,309] Gaining popularity, *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) is also used extensively for control of *Helicoverpa* species (cotton bollworm or corn earworm), which are serious global agricultural pests. Marketed commercially in multiple countries, HearNPV rapidly prevents insect host feeding and causes high levels of mortality. It also has utility in integrated pest management programs for protecting genetically engineered field crops from *Helicoverpa* species,[358] which have developed synthetic insecticide resistance. In 1975, the Environmental Protection Agency registered the first baculovirus as an insecticide in the United States. Other baculoviruses have also been registered and their implementation has been pursued by U.S. companies and more recently by those of other countries.[23,221,308] Commercial development of virus insecticides has been sporadic due in part to restricted markets and limitations in efficacy of these biopesticides, including kill speed. Nonetheless, because the baculoviruses are amendable to genetic engineering, progress has been made to improve insecticidal efficacy by the insertion of insect-specific toxin or hormone genes.[180,309] Carefully controlled field tests of genetically improved baculoviruses have been conducted in several countries, including the United States and Great Britain.[29,221,275] It is expected that viral insecticides will have an expanded role in integrated pest management programs as insects acquire resistance to chemical pesticides and environmental concerns increase. This practical application should expand with continued discovery and characterization of other virus families (ascoviruses, iridoviruses, tetraviruses, entomopoxviruses, nodaviruses, dicistroviruses, and cypoviruses) that are also potent pathogens of insects.

Description of Insect Virus Families

Baculoviridae

Classification of Baculoviruses

Members of the family *Baculoviridae* have a large, circular genome of double-stranded DNA that is packaged within an enveloped, rod-shaped virion.[316] The family name is derived from the Latin term "baculum" (stick or staff) to describe the shape of the virion. These viruses are characterized by their two morphologically distinct forms of infectious particles: occluded virus (OV) comprised of enveloped virions embedded within a crystalline matrix of protein and budded virus (BV) consisting of a single nucleocapsid surrounded by a plasma membrane–derived envelope. The family consists of four genera.[316] The alphabaculoviruses and betabaculoviruses infect lepidopteran insects (moths and butterflies), whereas the gammabaculoviruses and deltabaculoviruses infect hymenopteran insects (wasps and sawflies) and dipteran insects (mosquitoes), respectively.[275,341] The alpha- and betabaculoviruses include the nucleopolyhedroviruses (NPVs) and granuloviruses (GV), respectively; these two virus groups are the best characterized of *Baculoviridae*. For GVs, a single virion is embedded within a large ovicylindrical-shaped OV particle, which collectively appears as "granules" in the light microscope. By contrast, the polyhedral-shaped OV

particle (polyhedra) of the NPVs has numerous (>20) virions. The OV are produced and accumulate in the nucleus of NPV-infected cells, whereas in GV-infected cells, intracellular OV assemble after the nuclear membrane disintegrates.

Baculoviruses infect well over 500 different species of insects. To date, virus isolates have only been identified in arthropods, mostly from insects of the order Lepidoptera. The NPV type species is *Autographa californica* multicapsid nucleopolyhedrovirus (Ac*M*NPV), which was isolated originally from and named after the alfalfa looper *Autographa californica*. The GV type species is *Cydia pomonella* granulovirus (CpGV), isolated from the codling moth *Cydia pomonella*. Baculoviruses usually have distinct and relatively narrow host ranges that are limited to insects within a single genus or family. However, some baculoviruses, such as Ac*M*NPV, have broad host ranges that include >40 lepidopteran species.

In the next sections, the molecular biology and biochemical mechanisms of baculovirus multiplication are described with the intention to compare these unique viruses to the DNA viruses of vertebrates and to highlight the properties of baculoviruses that have been exploited for important applications in biology, biotechnology, and insect management. Recent reviews describe additional details of baculovirus biology, host and cellular interactions, evolution, host range, and pathology.[26,63,135,246,275,320,341]

Baculovirus Structure: OV and BV

Baculoviruses have a biphasic multiplication cycle in which BV is produced before OV.[275,341] Both virus forms (Fig. 19.1) are infectious and contain one or more rod-shaped nucleocapsids (30-60 nm in diameter, 250-300 nm long). The nucleocapsid contains a single covalently closed circle of double-stranded genomic DNA that is associated with a 6.9-kDa protaminelike core protein.[342] The outside proteins of the nucleocapsid interact to form ringlike subunits stacked every 4.5 mm; these include one major ~40-kDa capsid protein plus additional minor proteins.[343] The apical end of the virion has a distinct structure consisting of several unique proteins and suggesting a specialized function.[334] One of these virion end-structure proteins is pp78/83, a Wiskott-Aldrich syndrome (WASP)-like protein that contributes to actin-mediated propulsion of nucleocapsids within the cell and facilitates nuclear actin assembly; pp78/83 is essential for the production of infectious progeny virus.[110]

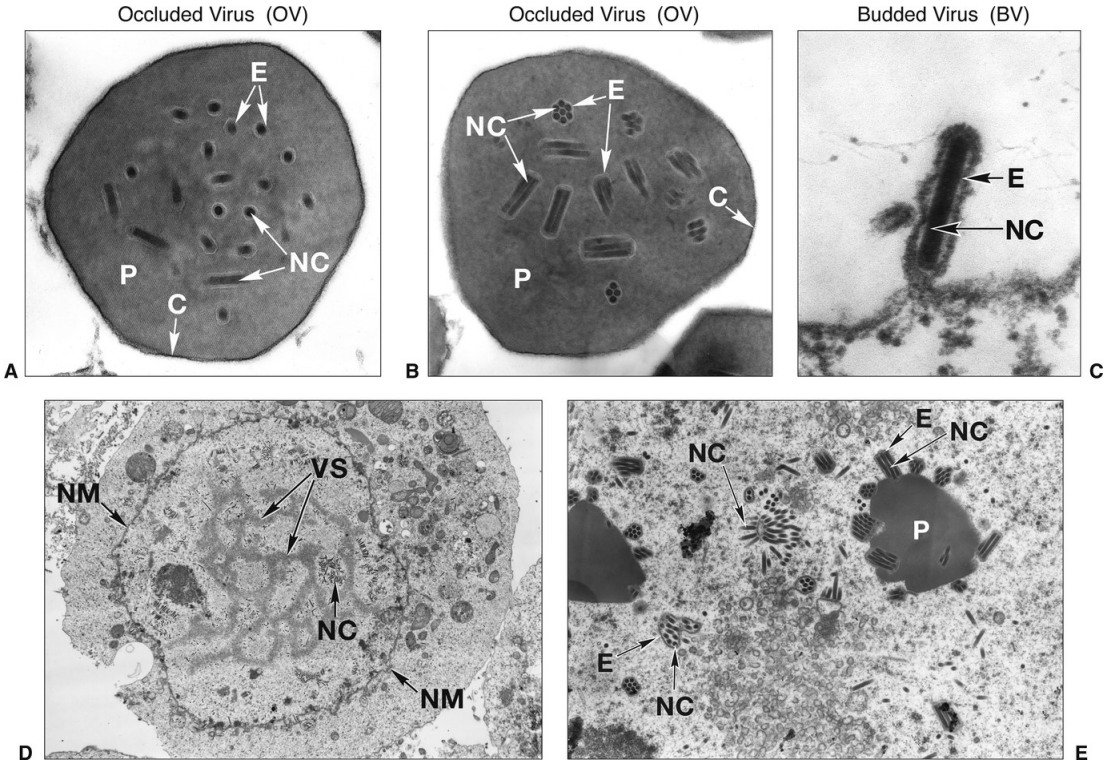

FIGURE 19.1 **Nucleopolyhedrovirus OV and BV particles**. **A**. Cross section of an OV particle from a single-capsid NPV (*S*NPV). Single nucleocapsids (NCs) are enveloped within a unit membrane (E) and embedded within a matrix of polyhedrin protein (P). The carbohydrate-rich calyx (C) covers the external surface of the particle, which is ~2-3 µm in diameter. **B**. Cross section of an OV particle from a multicapsid NPV (*M*NPV). The membrane envelopes (E) contain multiple nucleocapsids and are embedded within the polyhedrin matrix (P) of the particle. **C**. Budded virus (BV) particle during budding. The BV contains a single nucleocapsid (NC) (~30 × 250 nm) surrounded by a virus glycoprotein–containing envelope derived from the plasma membrane of the NPV-infected cell. **D**. Thin section of a typical cell in the late phase of *M*NPV infection. The virogenic stroma (VS) and associated nucleocapsids (NCs) are generated within the infected nucleus still contained by the nuclear membrane (NM). **E**. Enlarged thin section of an *M*NPV-infected cell nucleus early during the very late, occlusion-specific phase. Newly assembled nucleocapsids (NCs) associate with intranuclear membranes, are enveloped (E), and embedded within the polyhedrin matrix (P). (Electron micrographs were kindly provided by Dr. Malcolm J. Fraser Jr, University of Notre Dame.)

A distinctive feature of the baculoviruses is the large size (0.6-2 µm diameter) and polyhedral or oval shape of their OV particles (Fig. 19.1). The OV mediates virus transmission between insect larvae (caterpillar), which is the most susceptible developmental stage for infection. Upon ingestion, the OV protein matrix dissolves in the alkaline pH of the larva's midgut to release the occlusion-derived virions (ODV) that initiate infection of the midgut epithelium. The crystalline matrix of the OV is comprised primarily of a single ~29-kDa protein, designated either polyhedrin or granulin for the NPVs and GVs, respectively; these two proteins are closely related. The ODV of NPVs (Fig. 19.1A and B) are embedded in the matrix either as single nucleocapsids (*S*NPVs) or multiple nucleocapsids (*M*NPVs) that are surrounded by a single membrane

envelope. This nuclear-derived envelope contains virus-encoded proteins that are responsible for binding to midgut epithelial cells, the initial site of infection.[26,275,341] The membrane envelope is assembled *de novo* or represents a modified form of the nuclear membrane. On their surface, the mature OV particle has an additional covering of protein and carbohydrate known as the polyhedron envelope or calyx.

The BV form is produced prior to OV during infection. BV is responsible for cell to cell transmission within the infected insect. It is the infectious particle for inoculating cultured cells and thus is the form used for baculovirus-vector infections. The BV consists of a nucleocapsid surrounded by a loose-fitting membrane envelope that is acquired by budding from the plasma membrane of the host cell (Fig. 19.1C); it is, therefore, distinct from nucleocapsids embedded within OV. The envelope fusion protein (EFP) is the most abundant viral protein associated with the BV membrane. The NPVs are subdivided by their EFP type.[26,275,341] The EFP of group I NPVs (ie, Ac*M*NPV, Op*M*NPV) is GP64, whereas the group II NPVs (ie, Ld*M*NPV, Se*M*NPV) contain an F protein. GP64 is the best characterized of the baculovirus EFPs. It is a class III transmembrane glycoprotein that is required for efficient virus budding and BV infectivity[219]; BV can be neutralized by GP64-specific antiserum.[335,373] GP64 likely interacts with the host cell receptor or membrane components (still undefined in insects) and mediates membrane fusion upon activation by low pH in the endosome. Prefusion cleavage is not required. Detailed molecular studies[195,373] and the postfusion crystal structure[163] have revealed that GP64 is a stalklike, cross-linked trimer with an internal peptide domain comprised of fusion loops (similar to those of the vesicular stomatitis virus G protein) that are required for membrane fusion. However, the exact mechanism of GP64-mediated membrane fusion remains unclear. Because GP64 is essential for virus entry of mammalian cells during baculovirus-mediated gene transduction, it is used to facilitate entry of a variety of enveloped viruses into mammalian cells by using pseudotype strategies.[50,128,243,293]

Baculovirus Genome Structure

The baculovirus genome is a large (80-180 kbp) covalently closed, supercoiled circle of double-stranded DNA. To date, the complete nucleotide sequence of >170 baculoviruses, including viruses from each genus, has been reported. This abundance of genetic information has provided important clues regarding baculovirus functions, evolution, and host interactions.[275,341] There are ~30+ core genes that are shared by all baculoviruses and confer the distinctiveness to this unique family of insect viruses.[135,319] Certain conserved genes are essential for baculovirus transcription (during the early and late phases), viral DNA (vDNA) synthesis, vDNA packaging, and assembly of the OV and BV particles. Other core genes contribute specifically to baculovirus host range and the virus' unique life cycle within insects. Not surprisingly, certain genes are nonessential and thus dispensable for virus multiplication, especially in cell culture.

Ac*M*NPV is the best-studied baculovirus. Its 134-kbp DNA genome consists almost entirely of unique sequences that encode ~150 open reading frames (ORFs).[5,275] The ORFs are closely spaced on both DNA strands with no apparent organization with respect to temporal expression. Early, late, and very late genes are interspersed throughout the genome. Most ORFs are separated by only 2-200 nucleotides. These intergenic regions have a high adenosine/thymidine content and function as promoter or termination sequences. In addition, the translational termination codon (usually *UAA*) often overlaps the polyadenylation signal, AA*UAA*A. Multiple examples of overlapping ORFs exist. Interestingly, there are few introns in baculovirus genomes. A rare example of splicing involves the nonessential *ie*-0 gene and yields protein IE0 (see below), which is nearly identical to transcriptional activator IE1.[54]

Ac*M*NPV RNA transcripts often initiate from within or extend through adjacent genes, a phenomenon especially prevalent among viral RNAs late in infection.[51,94,205] Thus, numerous genes are transcribed into bi- or multicistronic RNAs wherein the upstream ORF is preferentially translated. The biological implication of this arrangement is unknown. There are also multiple examples of the transcription of overlapping and in some cases, antisense RNAs.[51] Thus, the interspersion of temporal classes of RNA transcripts may have functional or regulatory significance. For example, activation of the very late promoter of *polh*, the gene encoding the major structural protein (polyhedrin) of OV, downregulates overlapping, antisense RNAs initiated downstream of *polh*.[241]

A distinguishing feature of the baculovirus genome is the presence of multiple copies of interspersed repetitive sequences ranging in size from 30 to 800 bp that are designated homologous regions or *hr*s.[64,121] The *hr*s act as transcriptional enhancers[121,125] and probably function as origins of viral DNA replication.[170,251] Ac*M*NPV contains eight *hr*s, which account for 3%-4% of the genome. The *hr*s have a complex modular organization consisting of ~60-bp repeats, each with a highly conserved 28-bp imperfect palindrome (28-*mer*). The 28-*mer* is the minimal sequence required for orientation- and position-independent enhancement of promoter activity and DNA replication.[188,272] The *hr*s bind host- and virus-specific proteins. In particular, the transcriptional activator IE1 binds as a dimer to the palindromic 28-*mer* in an interaction that is required for IE1-mediated stimulation of enhancer activity.[174,188,273] Deletion of a single *hr* (either *hr1*, *hr1a*, *hr2*, *hr3*, *hr4a*, *hr4b*, or *hr5*) from the Ac*M*NPV genome reduces transcription of proximal genes but has no significant effect on vDNA replication or virus multiplication.[41,272] Sequential removal of all eight *hr*s from the AcMNPV genome compromised but did not eliminate virus production.[31] Whereas the *hr*s are not absolutely required for virus multiplication in cell culture, their functional significance remains unclear. Nonetheless, the novel distribution and repetition of the *hr*s or *hr*-like elements within the genome of multiple baculoviruses suggest that they have a critical role in the virus life cycle.

Stages of Baculovirus Replication

Primary and secondary infection

Baculovirus multiplication in the insect larva is divided into primary and secondary stages, which refer to the initial infection of larval midgut cells and the subsequent infection of secondary tissues, respectively (Fig. 19.2). Although virus binding to the host cell initiates infection in both stages, aspects of primary and secondary infections differ. Secondary infection in the larva (caterpillar) most closely resembles that of cultured cells. Most of the details of Ac*M*NPV replication (described below) have been obtained through studies of high multiplicity infection of cultured SF21 (or clonal SF9) cells, a convenient cell line derived from the nocturnal moth *Spodoptera frugiperda* (Lepidoptera; noctuidae). It should be appreciated that the timing of replication events often varies between viruses and insect hosts.

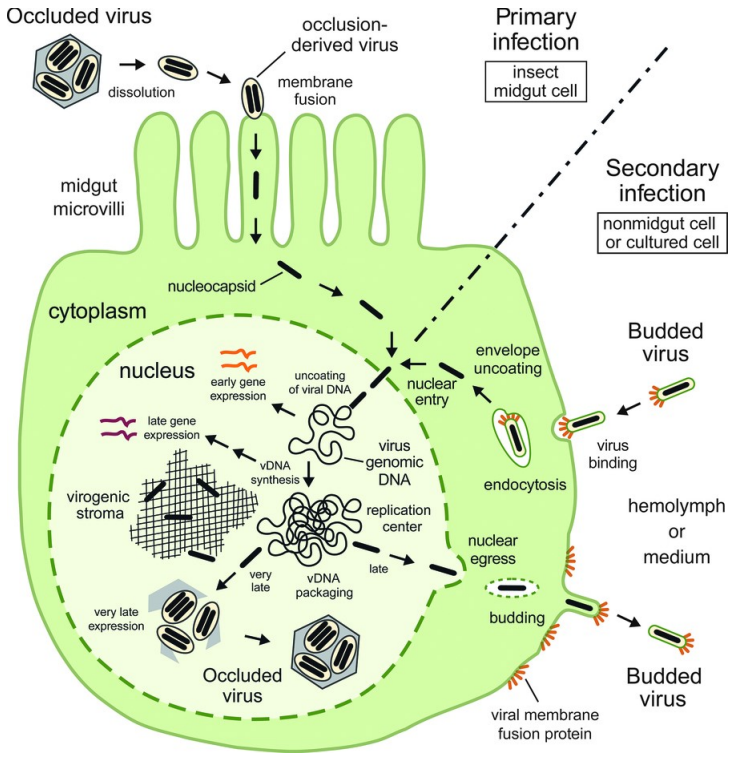

FIGURE 19.2 **Baculovirus multiplication cycle**. Except for the virus particle and the route of its entry, most steps in the baculovirus infection cycle are identical for an insect midgut epithelial cell (primary infection) or a subsequently infected nonmidgut cell (secondary infection). The steps for BV infection of a permissive cultured cell are the same as that for a secondary infection. Upon infection of a midgut cell by OV (*top*), the polyhedrin matrix of larval-ingested OV dissolves in the midgut lumen to release occlusion-derived virions that cross the peritrophic membrane and gain access to the columnar epithelial cells. These enveloped virions fuse with the microvilli membranes, thereby releasing nucleocapsids into the cytoplasm for nuclear entry or transport to the basal membrane side of the midgut cell for budding into the hemocoel or tracheal epithelium. In secondary infections (*right*), enveloped BV in the infected larva's hemolymph or growth medium from infected cell culture attaches to unidentified cell surface receptors and enters by endocytosis. As the endosome acidifies, the viral and endosomal envelopes fuse to release nucleocapsids into the cytoplasm. Occlusion-derived or BV-derived nucleocapsids both proceed to the nucleus where they interact with the nuclear pore. The viral DNA genome is released by uncoating and the early phase of transcription and gene expression begins. Once the appropriate early gene products appear, viral DNA is synthesized in association with the newly formed virogenic stroma. Late gene expression occurs after DNA replication begins and produces structural components for the nucleocapsid, which then package just-replicated viral genomic DNA. During this late phase, newly assembled nucleocapsids exit the nucleus, migrate to the plasma membrane, associate with an envelope fusion protein, and exit the cell by budding to yield infectious BV. By contrast, during the very late phase, nucleocapsids are retained in the nucleus, acquire a nuclear-derived envelope, and become embedded within a crystalline matrix of polyhedrin to yield OV particles. Mature OV are released by virus-mediated cell lysis. (The author acknowledges contributions of Dr. Nadine Dalrymple and Dr. David Taggart to this illustration.)

Attachment, endocytosis, and uncoating

Budded virus (BV) of Ac*M*NPV enters the host cell (Fig. 19.2) by what appears to be receptor-mediated endocytosis.[26,275,341] Although the host cell receptor(s) have historically eluded identification, recent evidence suggests the Niemann-Pick C1 (BmNPC1) cholesterol transporter contributes to entry of the *Bombyx mori* NPV.[196] For group I alphabaculoviruses, the predominant glycoprotein of BV, EFP GP64 is required for receptor interaction and envelope fusion of the endosomal membrane. EFP GP64 binds to BmNPC1.[196] Other studies suggest that GP64 interactions with the membrane-bound phospholipids may be the critical determinant for BV binding to the host cell.[26] After release within the cytoplasm, nucleocapsids migrate to the nucleus in a process that coincides with virus-induced actin polymerization.[48,110] Nucleocapsids interact end-on with the nuclear pore, enter the nucleus, and release their genome. Phosphorylation of the 6.9K protaminelike core protein by a virus-encapsidated protein kinase promotes DNA release.[342,356] These processes are rapid since new viral RNA transcripts can be detected within 15 min after inoculation.[54,126,177,226,317]

Early phase

By definition, the early phase of infection precedes virus DNA replication. During this period that extends to 6 hours, baculoviruses express genes encoding transcriptional activators, a virus-specific RNA polymerase activity, DNA replication factors, apoptotic suppressors, and other critical factors. These early gene products prepare the cell for the enormous burden imposed by the synthesis of viral nucleic acid and structural components that can constitute more than a third of the cell's mass (Fig. 19.3A and Fig. 19.5). The level and timing of early and late gene expression is orchestrated to insure proper temporal assembly of infectious BV and OV. Early viral gene products also block cell cycle progression forcing cells to accumulate within the S phase and G_2/M boundary.[34,154] In addition, filamentous actin and microtubules are redistributed, causing a dramatic rearrangement of the host cell cytoskeleton that is manifested by nuclear hypertrophy and cell rounding; these changes are characteristic of the early stages of baculovirus infection.[48,110,276]

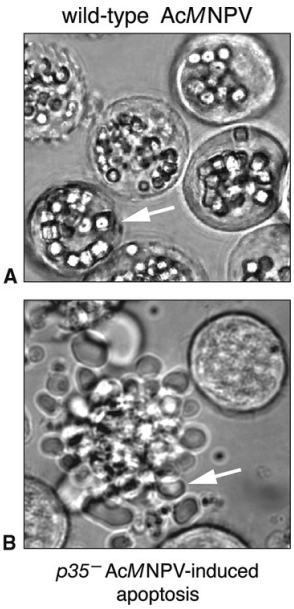

FIGURE 19.3 **Fates of baculovirus-infected cells**. **A**. Wildtype Ac*M*NPV infection of *Spodoptera frugiperda* SF21 cells. Upon successful inhibition of apoptosis by viral P35, the highly refractile OV particles (*arrow*) accumulate in the hypertrophied nucleus of each cell by 48 hours after infection. **B**. Late stages of apoptosis triggered by an Ac*M*NPV *p35*-deletion mutant. When virus-induced apoptosis is not suppressed, SF21 cells undergo widespread apoptosis that includes the formation of membrane-enveloped apoptotic bodies (*arrow*). (Magnification, 200×.) (Micrographs provided by Gulam Manji, University of Wisconsin-Madison.)

Baculovirus gene expression involves a highly coordinated cascade of gene activation events that is regulated primarily at the level of transcription.[51,93,275] Transcription of strictly defined early genes usually peaks between 6 and 12 hours after infection and declines thereafter as late viral transcription accelerates. During this early time, at least a portion of the incoming viral DNA genome adopts a nucleosomelike structure.[357] Early viral transcription is mediated by the host's RNA pol II.[24,119,146,152,266] Early promoters resemble host RNA pol II promoters, complete with a consensus TATA element located ~30 bp upstream from the RNA start site.[25,71,123,176,200,266,317] A tetranucleotide CAGT motif positioned at the RNA start site is common to many early promoters and functions as a transcriptional initiator element.[24,51,265] These core promoter elements also cooperate with upstream activating elements or more distal transcription enhancers (including the *hr*s) to interact with sequence-specific transcription activators. The *cis*-acting transcriptional control elements for multiple Ac*M*NPV early genes have been defined, including those for *ie-1*, *gp64* EFP, *pp31*, *he65*, *p143*, and *p35*. Many early promoters are highly responsive to virus-encoded transactivators, especially the multifunctional protein IE1 (see below). As expected, host factors also participate in early transcription events.[104,167,176,270] Remarkably, purified baculovirus genomic DNA devoid of protein is infectious. Thus, host factors are sufficient to initiate infection albeit at rate slower than that of infectious virus particles. This feature is exploited in the generation of the baculovirus expression vectors.[157,259,327]

Immediate early protein IE1 is the principal early transregulator of many baculoviruses. This nuclear phosphoprotein is highly conserved among the lepidopteran baculoviruses and is essential for virus multiplication by virtue of its transcriptional and DNA replication activities.[249,286,300,311] IE1 transactivates early baculovirus promoters and contributes to a critical but unknown virus DNA replication function.[25,123,124,175,179,200,226,248,249,271,311,317] Typical of transcriptional activators of DNA viruses, IE1 has a modular structure (Fig. 19.4). The N-terminal third consists of a potent transactivation domain that is largely acidic.[174,274,294] A small stretch of residues at the extreme N terminus of IE1 is required for viral DNA replication.[249,311] This N-terminal replication domain possesses phosphorylation sites within a highly conserved cdc/cyclin B kinase–like motif (TPXR/H) that are required for viral DNA replication and virus multiplication but not transactivation.[311] Thus, phosphorylation may serve as a molecular switch to ensure the proper regulation of IE1's transcriptional activities and its DNA replication duties in a temporally controlled manner. The responsible kinase remains to be identified. Ac*M*NPV IE1 contains a highly conserved patch of basic residues (designated basic domain I) within its N-terminal transactivation domain (Fig. 19.4) that is required for DNA binding.[238] DNA binding by IE1 is essential for virus multiplication.[310] IE1 binds as a dimer to the repetitive 28-bp palindromes that constitute the *hr*s; this DNA binding is required for optimal transcriptional enhancer function of the *hr*s.[121,125,174,188,226,272,273] The IE1 C terminus possesses a domain that also contributes to DNA binding (Fig. 19.4). Here, IE1 contains an oligomerization domain and a novel nuclear localization signal.[236,237] IE1 dimerization is required for nuclear import. Once in the nucleus, IE1 interacts with the *hr*s of the virus DNA genome as suggested by colocalization of IE1 and DNA replication factors and the requirement of basic domain I for virus multiplication.[224,234,310] IE1 is required for transient DNA (plasmid) replication assays and contributes to viral DNA replication.[249,269,271,311] It is likely that by binding to the *hr*s, IE1 recruits critical DNA replicative factors to the origins of viral DNA replication. However, the exact replicative functions of IE1 are unclear.

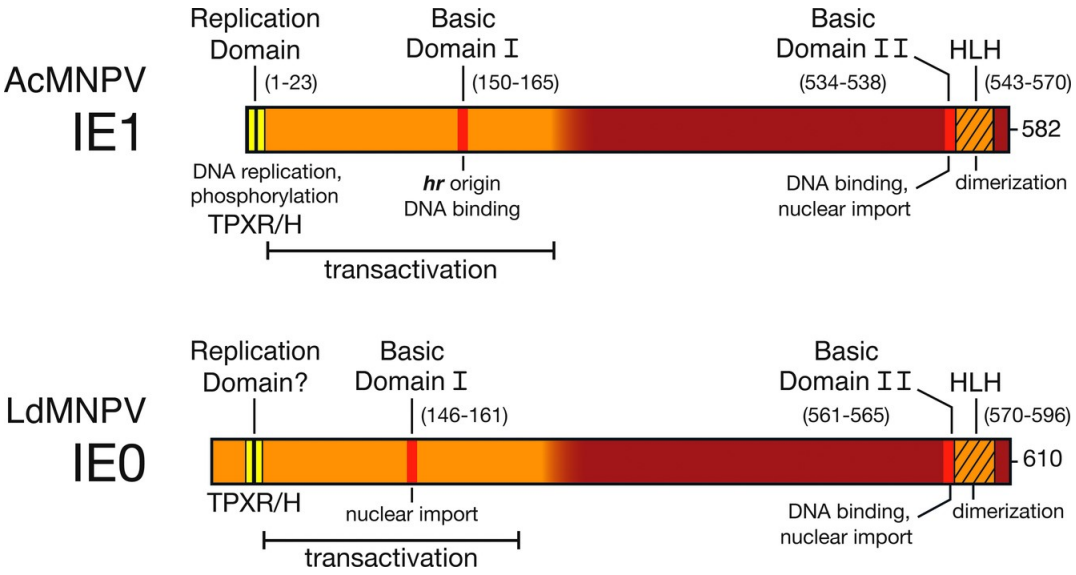

FIGURE 19.4 **Structure of baculovirus transactivator IE1**. NPV immediate early IE1 is a potent transcriptional activator essential for virus gene expression, DNA replication, and virus propagation. It has a conserved modular organization consisting of an N-terminal transactivation domain and a C-terminal helix-loop-helix (HLH) domain required for dimerization and nuclear import. The small basic domains (I or II) are required for DNA binding. IE1 from Ac*M*NPV and IE0 from Ld*M*NPV share structural organizations but have very divergent sequences. Both possess N-terminal TPXR/H motifs required for DNA replication and phosphorylation[311] but differ with respect to the function of their basic domains[68]; Ac*M*NPV IE1 basic domain I is required for *hr* DNA binding, whereas the same domain in Ld*M*NPV IE0 is required for nuclear import.

Baculoviruses encode additional transcription activators, including IE0, IE2, and PE38, which are conserved among the baculoviruses. Derived by RNA splicing, IE0 is identical to IE1 except for an additional 54 residues, encoded by *exon 0*, at its N terminus. IE0 has redundant regulatory activities with IE1 and is dispensable for virus replication.[54,175,203,273,300] Additional studies have indicated that IE0 preferentially transactivates certain viral genes at very early times in infection.[296] IE2 is a 47-kDa leucine zipper, RING finger–containing transactivator.[40,318,368] Ac*M*NPV mutants containing loss of function *ie-2* alleles are viable but exhibit delays in virus replication.[261] Upon overexpression in plasmid-transfected cells, *ie-2* causes cell cycle arrest,[262] but the significance of this activity is unknown. IE2 and the transregulator PE38 both localize to punctate nuclear structures in transfected cells.[178] PE38 is a promoter-specific transactivator with a leucine zipper and RING finger.[177,200,248] Ac*M*NPV *pe38*-deletion mutants exhibit reduced levels of DNA synthesis and BV production but are viable.[214]

Late phase

The late phase of the baculovirus infection cycle starts with viral DNA replication, which begins ~6 hours after Ac*M*NPV infection. The synthesis of new genomic DNA and the expression of late structural genes are necessary for production of BV, the predominant infectious form during this period. Late gene expression requires viral DNA replication. This dependence is illustrated by inhibiting viral DNA synthesis through use of a temperature-sensitive (*ts*) mutant in the essential helicase *p143* gene (see below) that subsequently blocks late gene expression and eliminates BV production.[111] Likewise, RNA silencing (RNAi)-mediated ablation of virus DNA replication factors prevents Ac*M*NPV DNA synthesis, blocks late viral transcription, and prohibits the characteristic shut-off of host protein synthesis.[285] The molecular basis for the coupling of late gene expression to DNA replication is unknown, but common among large DNA viruses.

At least six baculovirus genes, which include *p143*, *ie-1*, *lef-1*, *lef-2*, *lef-3*, and *dna pol*, are required for DNA replication (Table 19.2). These genes were originally identified using transient DNA replication assays conducted in cultured cells with plasmids containing a viral *hr* enhancer/origin.[168,202] Replication factors include a DNA binding helicase (p143), a putative primase (LEF-1), a primase-associated protein (LEF-2), a single-stranded DNA-binding protein (LEF-3), and a virus-encoded DNA polymerase (dna pol). The essential function of these replication genes during infection has been confirmed through the analysis of conditionally lethal mutants and deletion mutants generate by using *Escherichia coli*–replicating bacmid technology (Table 19.2). Besides its positive transcriptional activities, IE1 promotes origin (*hr*)-specific plasmid DNA replication.[168,202,269] A stretch of 23 residues at the IE1 N terminus is required for this activity.[249,311] IE1's capacity to act as a DNA origin (*hr*)-binding protein and to stimulate homologous recombination suggests that it has a direct role in DNA replication,[66,188,273] but its specific functions are still unclear. The *dna pol* gene[324] is also essential for Ac*M*NPV replication as demonstrated by the loss of virus viability upon its deletion.[330] Other genes, including *ie-2*, *lef-7*, *pe38*, and *p35*, directly or indirectly stimulate replication in transient replication assays.[201] Viral factors localize to centers of viral DNA replication within the virogenic stroma that forms within the nucleus (see below).[234] As these replication centers expand, host chromatin becomes marginalized to the inner edge of the nucleus; viral factors IE1, LEF3, and helicase P143 in the presence of a viral hr element are sufficient for this marginalization.[224]

TABLE 19.2 Baculovirus DNA replication and late expression factors

Virus Gene	Essential Gene[a]	DNA Replication	Late Expression	Function/Homology	Virus Mutants
ie-1	YES	+	+	Transcription transactivator, origin of DNA replication binding protein	*ts*B821
ie-2/ie-n	NO	+	+	Transcription activator, E3 ligase, cell line–specific cell cycle regulator	
p47	YES	–	+	Late RNA polymerase subunit	*ts*317
p143	YES	+	+	Helicase, DNA binding protein with ATP binding, host range determinant	*ts*8
dnapol	YES	+	+	DNA polymerase, 3′→5′ exonuclease, proofreading activity	
pp31/39K	NO	–	+	Virogenic stroma association, phosphoprotein, binds DNA	
p35	NO	+	+	Caspase substrate inhibitor, apoptosis suppressor, related to p49	
lef-1	YES	+	+	DNA primase activity, DNA binding, LEF2 association	
lef-2	YES	+	+	Primase-associated protein, amplifies DNA replication, L and VL expression	VLD1
lef-3	YES	+	+	ssDNA binding, p143 helicase association and nuclear import	
lef-4	YES	–	+	Late RNA polymerase subunit, 7′ methyl G-capping activity, 5′ triphosphatase	*ts*538
lef-5	YES	+	+	Late RNA polymerase subunit, transcriptional initiation, has TFIIS homology (Zn ribbon), highly conserved	
lef-6	NO	–	+	Involved in late gene expression, vaccinia virus RNA pol homology	
lef-7	NO	+	+	Cell line–specific DNA replication, modulation of the DNA damage response, SCF complex association	vlef-7-AG
lef-8	YES	–	+	Largest late RNA polymerase subunit, catalytic active site for RNA synthesis	*ts*S1
lef-9	YES	–	+	Largest late RNA polymerase subunit, part of the catalytic active site	
lef-10	YES	–	+	Prionlike activity	
lef-11	YES	+	+	Upregulates host ATPase to modulate energy metabolism	
lef-12	NO	–	+	TFIIA similarity, transcriptional initiation	
pe38	NO	+	+	Transactivator of helicase *p143*	
an	YES	+	–	Alkaline nuclease, 5′→3′ exo- and endo-nuclease, DNA maturation and packaging	
hcf-1	NO	+	+	Cell line–specific, protein synthesis shutdown, late and very late expression	
vlf-1	YES	–	+	Hyperexpression of very late genes, structural protein, integrase/resolvase, vDNA packaging	*ts*B837

[a]Gene is designated “essential” as determined by direct deletion or replacement within the Ac*M*NPV genome.

As with many large DNA viruses, the precise mechanisms of baculovirus DNA replication are poorly understood. The *hr* repeats are likely origins of viral DNA replication,[170,188,251,272] but this function has not been demonstrated yet. Non-*hr* sequences have also been implicated as genome replication origins.[169,187,361] During infection, multiple unit-length genome fragments and concatemers of viral genomes have been detected.[187,244] Although these potential replicative forms are consistent with a rolling circle mechanism, no obvious mechanism for DNA replication has emerged from various studies.[275] Experimental evidence also suggests that recombination-dependent DNA replication contributes to the generation of baculovirus genomic DNA. For example, all baculoviruses encode an alkaline nuclease (*AN*) gene with sequence similarity to that of the phage lambda *red* exonuclease, which is involved in phage DNA recombination. Deletion of the alkaline nuclease gene *AN* from Ac*M*NPV causes loss of virus viability even though viral DNA synthesis is normal.[235] *AN* contributes to production of longer than genome length DNA fragments in infected cells, a property consistent with a role in recombination. The endonuclease and 5′→3′ exonuclease activity of the alkaline nuclease may also participate in viral DNA maturation and packaging. Newly replicated, unit-length circles of DNA are packaged into individual nucleocapsids within the virogenic stoma.

Early in infection, one or more of the processes associated with baculovirus DNA replication is sufficient to trigger the host’s DNA damage response and apoptosis.[150,218,285] Baculovirus-encoded apoptotic suppressors (see below) are necessary to block premature cell death caused by apoptosis and to allow completion of the virus’ life cycle. Recent studies suggest that the host’s DNA damage response is necessary for optimum virus DNA replication.[150,218] For example, pharmacological inhibition or RNAi-mediated ablation of the host kinases that initiate the DNA damage response reduce Ac*M*NPV DNA replication and severely restrict virus production. Thus, baculoviruses likely modify specific aspects of the host’s DNA damage response for their own benefit.[218]

Nineteen baculovirus genes are required for late gene expression (Table 19.2), as first determined for Ac*M*NPV.[193,202,269] These genes, designated late expression factors (*lef*s), were identified by their ability to support high level expression of a reporter gene placed under control of a virus late promoter and *cis*-linked to an *hr* origin/enhancer element.[248] Due to the link between DNA replication and late gene expression, several genes required for late gene expression (*ie-1*, *p143*, *lef-1*, *lef-2*, *lef-3*, and *dnapol*) are essential DNA replication factors (Table 19.2). Because they are dispensable for late gene expression in certain cell lines, multiple *lef*s (*ie-2*, *lef-7*, and *hcf-1*) confer tissue or species specificity. In addition, a variety of viral genes contribute to host range, including helicase *p143* and host range factor-1 (*hrf-1*).[49,65] Several *lef*s function

directly or indirectly at the level of transcription or mRNA stabilization (Table 19.2). Of these genes, four (*p47*, *lef-4*, *lef-8*, and *lef-9*) encode components of the baculovirus RNA polymerase[127] that is specific for late and very late gene expression (see below). The roles of transcription-specific *lef*s during infection have also been characterized by using conditionally lethal mutations (Table 19.2).

The baculoviruses are unique among nuclear-replicating DNA viruses in that they switch to a novel RNA polymerase for late and very late transcription.[247,275] The virus-encoded RNA polymerase retains specificity for late virus promoters *in vitro* and is distinguished from other host RNA polymerases by its resistance to α-amanitin and tagetitoxin, which are inhibitors of RNA pol II and III, respectively.[108,364] The late RNA polymerase complex includes virus proteins p47, LEF-4, LEF-8, and LEF-9 as well as other proteins.[15,127] The viral-encoded polymerase recognizes and initiates transcription within a nucleotide motif (A/G/T)TAAG that functions as the primary sequence element for late and very late promoter activity. All genes encoding proteins that are synthesized in abundance during the late phase (eg, major capsid protein vp39, 6.9K basic core protein, gp64 EFP) possess this late TAAG motif.[51] Fewer than 18 nucleotides surrounding this initiation site are sufficient to direct high-level late gene expression.[220] Some late genes, including the *vp39* capsid gene, have multiple TAAG motifs, each of which serve as an independent RNA start site.[321] This redundancy boosts production of gene products needed in abundance for virion assembly. It is noteworthy that some early genes (*p35*, *gp64*, *pp31*, and *ie-1*) are also transcribed late in infection. Late transcription of these genes is often mediated by late TAAG motifs that overlap the early promoter elements.[25,123,226,265]

Very late phase

The final, very late stage of baculovirus infection is characterized by the hyperexpression of occlusion-specific genes and the production of mature OV from 18 through 76 hours after infection or until cell lysis (Figs. 19.3A and 19.5). The very late phase is marked by a dramatic increase in transcription of *polh*, which encodes the major matrix protein polyhedrin of OV. The very late hyperexpression of *polh* and its nonessential nature for BV production form the basis of the baculovirus expression vector system (see above). Foreign genes are commonly placed under control of the *polh* promoter replacing the *polh* ORF to obtain high levels of expression very late in infection of cultured cells.[44,157,327] The promoter for the very late, hyperexpressed *p10* gene (see below) has also been used.

The baculovirus-encoded RNA polymerase also mediates very late gene transcription. The promoters of the hypertranscribed genes (*polh* and *p10*) contain the late TAAG motif and are positively regulated by the 50 nucleotides constituting the noncoding leader of their gene.[247,275] This region is responsible for the very late burst of transcriptional activity that distinguishes very late genes from late genes. These "burst" sequences may interact with activation factors responsible for the stimulation of transcription at this late time. Host and virus-specific factors likely participate in transcription of very late genes.[247,275] Indeed, the baculovirus very late factor-1 (VLF-1) is a candidate for a positive activation. VLF-1 forms a complex with the "burst" sequences and stimulates very late transcription.[209,217,367] Interestingly, VLF-1 is an essential protein (Table 19.2); in the absence of VLF-1, very late gene expression is compromised and virus DNA accumulation and packaging is reduced.[329] Thus, VLF-1 may also contribute to viral DNA replication and nucleocapsid assembly.

p10 is the other very late, hyperexpressed gene of baculoviruses. It encodes a small 10-kDa coiled-coil domain protein that associates with expansive fibrillar structures (Fig. 19.5), which assemble in the nucleus and cytoplasm late in infection.[228] P10 is a nonessential protein that may disrupt the nuclear membrane or stabilize OV particles.[115,354]

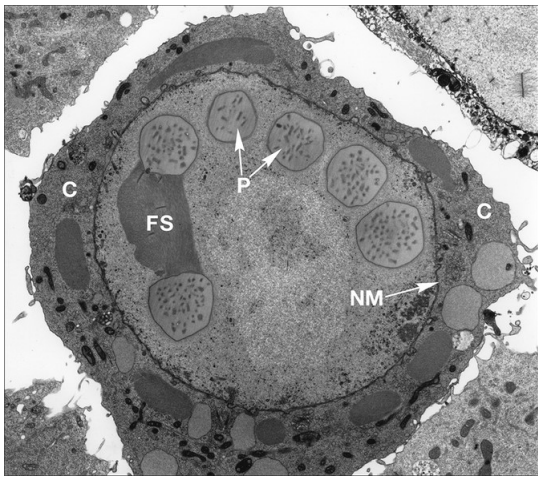

FIGURE 19.5 **The very late occlusion stage of Ac*M*NPV infection**. Multiple OV particles (P) form on the inside edge of the hypertrophied nucleus near the inner nuclear membrane (NM). Dense fibrillar structures (FS) often form within the nucleus. The characteristic swelling of the nucleus forces the densely packed cytoplasm (C) to the cell's edge. (Electron micrograph was provided by Dr. Peter Faulkner and Dr. Gregory Williams, Queens University.)

Virus assembly, budding, and occlusion

Nucleocapsids assemble during the late and very late phases of infection.[275,372] Newly assembled nucleocapsids appear at the edge of the stromal matte located in the virogenic stroma (Fig. 19.1D), a poorly understood viral nucleoprotein structure centered within the nucleus. Consisting of DNA, RNA, and virus protein, the virogenic stroma is the likely site of viral DNA replication, condensation, and encapsulation.[89,369] As infection proceeds, the viral replication center (virogenic stroma) expands and host chromatin becomes marginalized to the nuclear periphery. This process involves the combined activities of the *ie-1*, *lef-3*, and *p143* genes and requires the presence of an *hr* element.[224] Baculovirus phosphoprotein pp31 is a principal component associated with the virogenic stroma; it binds DNA by a phosphorylation-dependent mechanism, but its exact function is unclear.[35,122] pp31 appears to stimulate viral transcription rather than

regulating vDNA replication.[366] Newly assembled nucleocapsids exit the nucleus by budding or transport through nuclear pores (Fig. 19.2).[275,372] Nuclear actin plays a critical role in the maturation of these nucleocapsids.[48,110,233] The nuclear envelope is removed during transport to the plasma membrane. By interacting end-on with the plasma membrane, single nucleocapsids bud from the cell surface (Fig. 19.1C) and acquire the envelope fusion protein EFP GP64.[242] The rate of BV release from Ac*M*NPV-infected cells increases exponentially from 10 to 20 hours and then declines through 36 hours.

Late in infection, OV particles assemble and accumulate in the nucleus of infected cells (Figs. 19.3A and 19.5). For Ac*M*NPV, OV production begins about 18-24 hours after infection and accelerates as production of BV declines. Not surprisingly, OV assembly involves a multitude of virus genes and processes, including virus modification of nuclear membranes and vigorous import of polyhedrin into the nucleus.[275] Nucleocapsids align along newly assembled membrane segments in the nucleus and subsequently acquire an envelope prior to encasement by polyhedrin (Fig. 19.1E). Studies of the generation of this nuclear membrane envelope and nuclear import of critical membrane proteins have revealed new facets to integral membrane protein trafficking.[33] OV formation is first observed on the inside edge of the nucleus (Fig. 19.5). Time-lapse video microscopy has suggested that the number of OV per cell is established early, and with time each particle increases in size.[207] Maturation of the OV includes deposition of the polyhedron envelope (calyx) over the particle's surface. Finally, OV are released upon disintegration of the nucleus and loss of plasma membrane integrity very late in infection.

Disease Progression in Insects

Horizontal transmission of baculoviruses is mediated by the OV, the form of the virus that is most stable and resistant to environmental degradation. Primary infection occurs in the midgut epithelium (Fig. 19.2) of the insect larva.[275] Infection is initiated by larval ingestion of virus-contaminated food. Upon entry in the midgut lumen, the OV particles dissolve in response to the alkaline environment and release occlusion-derived virions (ODV). Passage of virus through the peritrophic membrane (Fig. 19.2) is facilitated by virus-encoded proteases (called enhancins), first discovered in the GVs.[189] ODV subsequently bind to the brush border membrane of columnar epithelial cells and enter by direct fusion with the plasma membranes (Fig. 19.2). Infection proceeds by pathways comparable to that in cultured cells with the interesting exception that newly synthesized EFP GP64 is transported unidirectionally to the basal membrane side of the midgut cells.[246] This targeting of GP64 allows nucleocapsid budding at the basal lamina and promotes rapid access of enveloped virus to the hemocoel and tracheal epithelium for systemic spread. Indeed, reduction of early GP64 synthesis selectively lowered the *per os* virulence of an Ac*M*NPV mutant,[349] suggesting an important role for this directional transport. The production of OV in these midgut cells is rare for lepidopteran hosts. However, multiplication of certain baculoviruses, including mosquito NPVs, is restricted to the midgut. Thus, OV is produced there.

BV first generated in the midgut is next spread through infection of hemocytes (blood cells) and the epithelial cells that line the tracheal network.[246] Interestingly, the lepidopteran baculoviruses encode v*fgf*, which bears sequence similarity to fibroblast growth factor. Recent studies with Ac*M*NPV suggest that v*fgf* activity in infected midgut cells activates extracellular metalloproteases that permeabilize the basal lamina, attracts susceptible tracheal cells, and allows BV to rapidly gain access to the trachea network and hemocoel.[211] This secondary infection affects virtually all internal tissues of highly susceptible larva. Within several days, the larva is converted into a milky white liquid, consisting mostly of OV particles. This process, referred to as "melting" or "liquification," is facilitated by a virus-encoded chitinase and a cathepsin, which cooperate to breakdown the larval cuticle (skin) that includes chitin.[139,144,145] The activation and secretion of these enzymes is carefully regulated so as not to prematurely destroy infected cells or the larva before virus production is maximized.[144,145] A typical Ac*M*NPV infection produces over 10^8 OV particles, a yield that constitutes >10% of the dry weight of the insect. Upon rupture of the cuticular exoskeleton, OV are liberated into the environment. Liquification represents the successful conclusion to the baculovirus life cycle as the release of OV is required for virus dissemination through the host population. Nonetheless, there is limited evidence that baculoviruses establish persistent infections in some hosts.[275]

Baculovirus-Mediated Alterations of the Host

Besides causing mortality, baculoviruses can exert dramatic effects within their host. At the cellular level, infection induces major changes in the cytoskeleton and nucleus of infected cells. Transcription of most host genes is significantly reduced in Ac*M*NPV-infected cells.[240,278] Nonetheless, certain host proteins used by the virus are upregulated, including heat shock proteins, membrane trafficking proteins, and TATA-binding proteins.[268,278] In general, host protein synthesis declines rapidly after the onset of baculovirus DNA synthesis. When Ac*M*NPV DNA replication is blocked, virus-mediated arrest of host protein synthesis is prevented.[285] Conversely, when Ac*M*NPV late gene expression is selectively blocked, arrest of host protein synthesis is unaffected. Because vDNA replication also triggers Ac*M*NPV-induced apoptosis (see below), the shutoff of host protein synthesis and apoptosis may be caused by the same host cell response to vDNA synthesis. The biochemical mechanisms involved are not yet understood.

Remarkably, the baculoviruses also exert control of their hosts at the physiological and behavioral level as a means to enhance virus production and dissemination.[228,320] For example, baculovirus-infected larvae often display a classical behavior of wandering that involves climbing to the top of vegetation, where they succumb and liquefy while hanging from a leaf or branch. This strategy facilitates virus dispersal by wind and rain. A baculovirus-encoded protein tyrosine phosphatase gene (*ptp*) contributes to this virus-induced behavior.[165] Uninfected caterpillars in late stages of development also wander. Because this behavior is controlled by a cellular *ptp*, it is postulated that baculoviruses acquired a host ancestral *ptp* because of the conferred selective advantage for dissemination. Recent studies have revealed another fascinating virus-mediated alteration of the host's visual perception at the levels of photoreception and phototransduction that serves to induce vertical climbing in host larvae and thus enhance virus dissemination.[198] Baculoviruses block development of their larval hosts by controlling the activity of ecdysone, the steroid hormone that triggers molting.[229] The baculovirus *egt* gene encodes an ecdysteroid UDP-glucose/galactose transferase that is secreted from infected cells and transfers glucose or galactose to these ecdysteroids, thereby inhibiting uptake of the hormone by cells. Virus-encoded *egt* blocks larval molting,[228,320] which is correlated with increased virus production; indeed, *egt* deletion mutants cause early mortality and yield less virus.[230] The increased speed of kill of *egt* deletion mutants has already been adapted for the generation of engineered baculoviruses for improved insecticidal activity. Interestingly, the inhibition of molting by baculovirus *egt* is also associated with enhanced wandering (climbing) of gypsy moth (*Lymantria dispar*) caterpillars upon infection with *Lymantria dispar* NPV.[147] The extent of this effect depends on virus dose and the developmental stage at the time of inoculation.

Baculovirus Manipulation of Apoptosis

The remarkable replicative success of baculoviruses often depends on their capacity to suppress host cell apoptosis, a dynamic suicide response that can function as an effective antiviral defense. As such, the baculoviruses have provided a wealth of information on the mechanisms by which viruses impede host apoptosis (also referred to as programmed cell death) to facilitate their multiplication.[17,58] These DNA viruses have revealed highly conserved genes that regulate apoptosis in insects and mammals alike. Apoptosis is critical for many normal processes in animals, including development, differentiation, immune function, and elimination of damaged, oncogenic, or pathogen-infected cells. Thus, misregulation of apoptosis is associated with many disease states.[99,337] By virtue of the discovery of multiple baculovirus genes that function as potent suppressors of apoptosis in phylogenetically diverse organisms, these viruses have provided valuable insight and important tools for studying the pathways by which apoptosis is either suppressed or amplified in human diseases and how apoptosis can be manipulated in treatment regimens thereof.

Baculovirus mutants that lack suppressors of apoptosis trigger widespread, species-specific cell death.[60,61,141,164] In some cases, apoptotic death approaches 95% of the infected cells within a 24-hour period, a response level that is unmatched by mammalian viruses. Baculovirus-induced apoptosis is characterized by premature cell death that includes activation of host prodeath components, fragmentation of host and viral DNA, and dismemberment of the cell into extracellular vesicles called apoptotic bodies (Fig. 19.3B). Accordingly, apoptosis severely limits production of virus. In the case of Ac*M*NPV mutants deficient in apoptosis suppressors, virus yields are reduced as much as 10 000-fold in a cell-specific manner.[61,141] Moreover, the infectivity of these mutants in certain permissive insects (ie, *Spodoptera frugiperda*) is 25-1000 fold lower than that of wild-type virus.[61] This reduced infectivity is correlated with increased apoptosis in larvae and reduced virus spread.[57] Thus, apoptosis impedes baculovirus multiplication and can function as an effective antivirus defense in insects.

Cultured insect cells have proven invaluable in studies of baculovirus-induced apoptosis. In particular, lepidopteran *Spodoptera frugiperda* (SF21) cells and dipteran *Drosophila melanogaster* (DL-1 or S2) cells have been used as model systems for these studies and those of invertebrate apoptosis.[43,60,61,149,184-186,207,223,285,286,290] Many of the cellular components comprising the apoptotic pathways of these cells have been identified. Moreover, these cells are sensitive to diverse apoptotic stimuli, including that caused by viruses, DNA damaging agents, transcription and protein synthesis inhibitors, calcium ionophores, phosphatase inhibitors, and others (Fig. 19.6A). Nonetheless, baculovirus-permissive cell lines refractory to virus-induced apoptosis have been identified. For example, cultured *Trichoplusia ni* TN368 cells fail to undergo apoptosis upon infection with Ac*M*NPV mutants that cause apoptosis of SF21 cells.[61,141] The infectivity of these mutants in *T ni* larvae is comparable to that of apoptosis-suppressing wild-type virus. The molecular basis of this interesting resistance to virus-induced apoptosis is unknown.

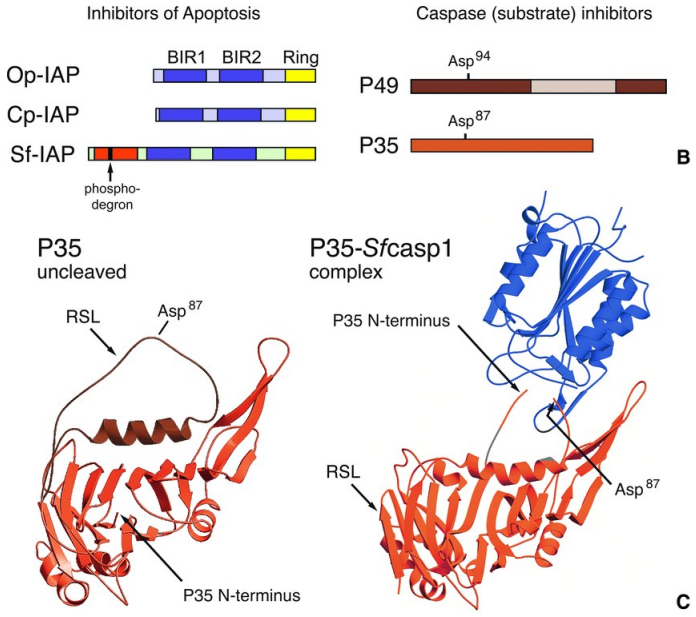

FIGURE 19.6 **Baculoviruses and apoptosis**. **A**. Model pathway for virus-induced apoptosis. Ac*M*NPV triggers widespread apoptosis of model *Spodoptera frugiperda* (order Lepidoptera) cells due to events associated with virus DNA replication. Such replication triggers a DNA damage response, which produces an apoptotic signal that depletes host cell IAP (cIAP). Loss of cIAP activates initiator and effector caspases, which if uninhibited cause proteolysis and apoptotic cell death. Virus-induced cIAP antagonists may be prodeath factors, like HID and reaper, activated host kinases, or the arrest of protein synthesis that accompanies baculovirus infection. Virus-encoded apoptotic suppressors produced early in infection prevent apoptosis. P35 inhibits active caspases, including *Sf*-caspase-1, whereas P49 inhibits an activated initiator caspase, probably S*f*Dronc. Baculovirus-encoded IAPs block procaspase activation at an unknown (?) step upstream of P49. **B**. Baculovirus apoptotic regulators. The IAPs contain two BIRs motifs and a C-terminal Ring finger motif. The viral IAPs, Op-IAP and Cp-IAP, closely resemble SfIAP, a cellular IAP from *S. frugiperda*. Caspase inhibitors P49 and P35 are 49% identical and contain aspartate residues (Asp[94], respectively and Asp[87]) required for caspase cleavage. P49 contains a 120-residue insertion (pink) unrelated to P35. **C**. Caspase inhibition by baculovirus P35. In uncleaved P35 (*left*), the prominent reactive site loop (RSL), with the Asp[87]caspase cleavage site at the apex, is solvent exposed while the N-terminus is buried. During cleavage, P35 undergoes a dramatic conformation change (*right*) in which the RSL folds backward and the N-terminus is liberated so that it enters the active site of the targeted caspase, forming a stable, inhibited complex.

Baculovirus-encoded apoptotic suppressors

To counteract the host suicide response and thus promote multiplication, baculoviruses have evolved novel apoptotic suppressors. These viral proteins regulate apoptosis at different steps in the death pathway (Fig. 19.6A) and in a surprising diversity of organisms. Among different baculovirus species, two mechanistically distinct apoptotic suppressors have been identified: the caspase inhibitors (P35 and P49) and the IAP proteins (Fig. 19.6B). The discovery of cellular IAPs with structural and biochemical properties comparable to those of viral IAPs suggests that baculoviruses originally acquired these apoptotic regulators from their insect hosts.[43,151,289] The same is most likely true for the baculovirus-encoded caspase inhibitors, but cellular homologs have not yet been reported. The strong selective advantage conferred to baculoviruses by expression of an apoptotic suppressor, like P35,[191] is consistent with the acquisition and maintenance of such host genes. Interestingly, a single functional regulator per virus appears sufficient. For example, Ac*M*NPV encodes one copy of the *p35* gene along with two nonfunctional *iap* genes.[62,116] By contrast, Op*M*NPV encodes a single functional *iap* (Op-*iap3*), three nonfunctional *iap*s, and no *p35*.[22,210]

Baculovirus P35 is a potent caspase inhibitor, often considered a universal suppressor of apoptosis due to its function in phylogenetically diverse organisms. First discovered in Ac*M*NPV,[60] *p35* prevents programmed cell death in plants, nematodes, flies, moths, and mammals (human cells included), among others, when expressed ectopically.[17,58] Accounting for its widespread effectiveness, P35 (35 kDa) is a broad-specificity substrate inhibitor of the effector caspases, which are the proteases that execute the cleavages that dismember the apoptotic cell.[39,99] P35 is cleaved by its target caspase at a requisite aspartate residue (Asp[87]) and forms a stable, stoichiometric complex with it.[16,36,374] The 2.2-Å crystal structure of P35 reveals that the Asp[87] cleavage site is located at the apex of a novel solvent-exposed reactive site loop (Fig. 19.6C), the orientation of which is critical to P35's anticaspase activity.[86,376] Caspase-mediated cleavage induces a dramatic conformational change in the reactive site loop that stabilizes the covalent link between P35 and the caspase (Fig. 19.6C) in a novel mechanism for substrate-protease inhibition.[69,365] Baculovirus P49 is a related substrate inhibitor of the caspases. First identified in *Spodoptera littoralis* NPV,[77] P49 exhibits 49% amino acid identity with P35 (Fig. 19.6B) and likewise possesses a reactive site loop that is cleaved at a requisite aspartate residue (Asp[94]) by the participating caspase. Interestingly, P49 targets apical initiator caspases in permissive insect cells. Thus, P49 inhibits the caspase(s) that proteolytically cleave and activate the effector caspases that are inhibited by P35.[130,253,377] This target specificity is conferred in part by the cleavage site residues of P49.[130,377] Thus, baculoviruses have evolved multiple caspase inhibitors with different specificities. A functional P35-like caspase inhibitor is also encoded by the insect entomopoxviruses.[212]

The baculovirus IAPs were the first discovered members of the larger IAP family resident in diverse organisms and their viruses. The cellular IAPs are highly conserved regulators of cell fate that function during cell stress, immunity, tumorigenesis, and pathogen invasion.[59,99,131] In insects, these short-lived (~30 minute half-life) cellular proteins regulate the caspases, among other death components. The IAPs are defined by the presence of one or more essential baculovirus IAP repeat (BIR) domains (~80 residues), consisting of a conserved Zn^{+2}-coordinating arrangement of Cys and His residues (CCHC). Many IAPs also possess a critical C-terminal RING domain, which functions as an E3-ubiquitin ligase. The antiapoptotic activity of some, but not all, IAPs is derived from the ability of the BIRs to bind directly to and neutralize initiator and effector caspases through ubiquitination by the RING. During apoptosis initiated by certain signals, the BIRs also interact with proapoptotic IAP binding motif (IBM) proteins, which bind to and dissociate the IAP-caspase complex to liberate active caspases and execute apoptosis.[59,99,131]

Several insect virus families, including the baculoviruses, entomopoxviruses, iridoviruses, African swine fever virus, and the nudiviruses, encode functional and nonfunctional IAPs.[17,58] Of these, the baculovirus IAPs are the best characterized. The viral IAPs share many structural and biochemical features with the cellular IAPs.[38,43,151] Despite these striking similarities and their potent antiapoptotic activity, the viral IAPs use a mechanism that does not involve direct inhibition of active caspases. The best-studied viral IAP is Op-IAP3 from *Orgyia pseudotsugata* NPV.[22,38,360] This small 268-residue IAP contains two BIRs and a C-terminal RING (Fig. 19.6B); each of these motifs is required for antiapoptotic activity.[149,359] Upon its overexpression, Op-IAP3 blocks apoptosis triggered by various signals in cells from insects and mammals, consistent with a broad, conserved mechanism.[58] In baculovirus-infected *Spodoptera* cells, Op-IAP3 functions upstream of baculovirus caspase inhibitors P49 and P35 and thus likely inhibits initiator caspase activation.[208,377] Consistent with these findings, Op-IAP3 blocks the proteolytic activation of *Sf*-caspase-1 by an upstream initiator caspase.[185,208,288] However, unlike host insect IAPs, viral Op-IAP3 fails to bind and inhibit active caspases.[360] The Op-IAP3 BIRs interact with *Drosophila* proapoptotic IBM proteins, including Reaper, Hid, and Grim.[338,339] The relevance of these interactions is unknown since Op-IAP3 fails to block apoptosis in *Drosophila*.[360,377] Importantly, the Op-IAP3 BIRs mediate homo- and hetero-oligomerization, which is also necessary for functionality of lepidopteran cellular IAPs.[43,149] Recent studies indicated that Op-IAP3 interacts with the cellular IAP of the permissive host *Spodoptera frugiperda*, SfIAP, and that this protein-protein interaction preserves the antiapoptotic function of SfIAP through a novel stabilization mechanism.[38] Without cellular SfIAP, Op-IAP3 fails to prevent apoptosis, indicating that Op- IAP3 requires SfIAP as a cofactor.[38] These findings suggest a new model wherein Op-IAP3 interacts directly with SfIAP to maintain its intracellular level, thereby suppressing virus-induced apoptosis indirectly.

Baculovirus-induced signaling of apoptosis

The host cell signal for apoptosis originates early in baculovirus infection. A critical link between virus DNA replication and the initiation of apoptosis has been established. Host caspase activation is one of the earliest events in execution. Proteolytic activation of the proform of *Sf*-caspase-1, the principal executioner caspase in the host *Spodoptera frugiperda*, coincides with the initiation of virus DNA replication.[184,185,208,288] Consistent with this finding, caspase-mediated cleavage of substrate inhibitors P35 and P49 is first detected when Ac*M*NPV DNA synthesis begins from 6 to 9 hours after infection.[16,377] P35 must be synthesized early before vDNA replication to effectively block virus-induced apoptosis.[142] Thus, apoptotic signaling occurs early before or at the start of viral DNA replication. Studies using vDNA polymerase inhibitors and conditional Ac*M*NPV mutants defective for vDNA synthesis indicated that vDNA replication or viral late gene expression was required to trigger apoptosis.[62,184] However, subsequent studies using RNA silencing for selective ablation of Ac*M*NPV factors required for vDNA replication or late gene expression demonstrated that vDNA replication, but not late gene expression, is required to trigger apoptosis.[285,286] Thus, like vertebrate DNA viruses, baculovirus DNA replication events are sufficient to trigger the apoptotic response.

Recent studies have uncovered important facets by which baculovirus DNA replication triggers apoptosis. In invertebrates, the short-lived cellular IAPs are central regulators of apoptosis. In cell lines of dipteran and lepidopteran origin, a threshold level of IAP is necessary to block constitutive signaling toward apoptosis. Thus, when intracellular IAP drops below this level, caspases become active and apoptosis ensues. Coincidental with the beginning of vDNA synthesis, Ac*M*NPV causes a dramatic loss of cellular IAP that forces caspase activation.[332] This IAP loss is independent of caspase activity and is mediated by residues within the N-terminal leader of the cellular IAP (eg, SfIAP) and the ubiquitin

ligase activity encoded by its C-terminal RING domain.[43,332] The N-terminus of SfIAP and multiple lepidopteran IAPs possess a conserved phosphodegron, which upon activation by phosphorylation causes rapid loss of the IAP by a ubiquitin-mediated pathway.[331] SfIAP's phosphodegron contains conserved mitogen-activated kinase (MAPK)-like regulatory sites suggesting that the phosphodegron responds rapidly to a signal-activated kinase cascade, possibly triggered by DNA damage.

The vDNA replication *lef*s of Ac*M*NPV are required for the virus-induced loss of cellular IAP. Thus, vDNA replication likely triggers a host cell response that depletes cellular IAP and executes apoptosis, likely through kinase activation. DNA replication of vertebrate viruses is a potent inducer of the host's DNA damage response, which subsequently triggers apoptosis.[353] Thus, it is likely that baculovirus DNA replication is interpreted as DNA damage, which is a potent cause of apoptosis in insects as well. Indeed, Ac*M*NPV DNA replication induces a DNA damage response in lepidopteran and dipteran cell lines.[150,218] Additional study is required to determine the link between the DNA damage response and phosphorylation-mediated depletion of cellular IAP in baculovirus-infected cells.

Host Transposons

The baculoviruses are one of the few families of animal viruses that accommodate the frequent and spontaneous insertion of host-derived transposable elements.[92,199,275] By virtue of their replication in the transposon-rich insect cell nucleus and the flexibility with respect to size of their DNA genome, baculoviruses are highly vulnerable to transposon insertion. This novel property has led to the identification of new families of transposons, which have been further developed as vectors for stable transformation of insects. It is likely that baculovirus evolution has been significantly influenced by arthropod transposons, which represent potent insertional mutagens that generate genetic diversity and provide new genes and genetic regulatory sequences to recipient viruses.[133,199,275]

Baculovirus mutants with transposon insertions are often distinguished by altered plaque morphology, including the "few polyhedra" (FP) phenotype in which reduced nuclear accumulation of OV facilitates mutant identification by light microscopy. Several FP-causing lepidopteran transposons, including PiggyBac, IFP2.2, IFP1.6, TFP3, and hitchhiker, have been identified by their insertion within or near the 25K protein gene (Ac61) of Ac*M*NPV.[12–14,42,90,91] Loss of function Ac61 mutants have reduced polyhedrin gene transcription and limited intranuclear envelopment of nucleocapsids that lowers OV accumulation.[13,32,138] The most prevalent baculovirus-associated transposons possess terminal inverted repeats flanking a transposase gene with specificity for the sequence TTAA. For example, PiggyBac is a small (2.47 kb) autonomous transposon identified in the moth *T. ni*.[90] It was the first nondrosophilid transposon used for stable germline transformation. PiggyBac and other related transposons have now been used for gene transfer in Lepidoptera, Diptera, Coleoptera, and Hymenoptera, and most importantly in insect species (ie, mosquitoes) of medical and agricultural significance for which P-element transformation has not been possible.[133,227,279]

The retrotransposon TED (transposable element D) was also discovered as an insertion within Ac*M*NPV.[92,95,213,216] TED is a 7.5-kb middle-repetitive retrotransposon that is actively transcribed within the Ac*M*NPV-permissive host *T ni*. Classified as an errantivirus (family *Metaviridae*), TED has a retroviruslike organization with *gag*, *pol*, and *env* genes that are flanked by long terminal repeats. It is closely related to errantiviruses of other lepidopteran and dipteran (*Drosophila*) insects.[95,213] Like a functional retrovirus, TED assembles viruslike gag-containing particles, complete with protease and reverse transcriptase activities.[132,190] The TED *env* gene also encodes a membrane-associated glycoprotein with properties expected of a retroviral envelope protein.[245] Interestingly, the *env* gene of TED and other errantiviruses bear a striking similarity to the baculovirus group II envelope fusion (F) proteins. Thus, it has been suggested that lepidopteran errantiviruses acquired their *env* gene from baculoviruses through an ancient integration/excision event.[206,252] Not ruled out is the alternative possibility that baculoviruses have acquired *env* (F) genes through errantivirus insertion, as is the case for the Ac*M*NPV insertion mutant carrying TED.[95,216] Nevertheless, the novel association between baculoviruses and active transposons has also led to studies demonstrating that virus-mediated shuttling of mobile genetic elements between insect species occurs in nature.[76,92,106,199,275]

Polydnaviridae (*Polydnaviriformidae*)

Classification and Structure of Polydnaviruses

The polydnaviruses have been historically classified as novel DNA viruses, which have their replication genes embedded within the genome of their insect host, the endoparasitic wasps. This novel arrangement allows for gene expression strategies that provide beneficial activities to the host wasp by facilitating development of the wasp's progeny within a parasitized host organism. However, these viruses do not replicate outside of the host wasp as they do not package a replicative genome or replication-specific gene products into their viruslike particles. Thus, as molecular studies of these viruses, the wasp host, and related virus families have progressed, debate about their classification as either viruses or viriforms (replication-incompetent gene transfer agents) have intensified. Recently, the International Committee on Taxonomy of Viruses (ICTV) ruled that members of the family *Polydnaviridae* are to be considered viriforms in classification and nomenclature and thus should be designated *Polydnaviriformidae*. For purpose of discussion here, these agents (polydnaviruses) will be referred to as "domesticated" endogenous viruses[75] (see below).

The polydnaviruses are large DNA viruses that are distinguished by their beneficial activities for their insect host, the endoparasitic wasps (order Hymenoptera). These wasps lay their eggs in the body of a living lepidopteran larva, wherein the wasp's offspring hatch and develop. Polydnavirus DNA-containing particles accompany the wasp egg upon oviposition and subsequently express virus-encoded virulence genes, which compromise the caterpillar's immune response and thereby protect and facilitate development of the wasp larva (Fig. 19.7). These viruses do not replicate in the parasitized caterpillar.

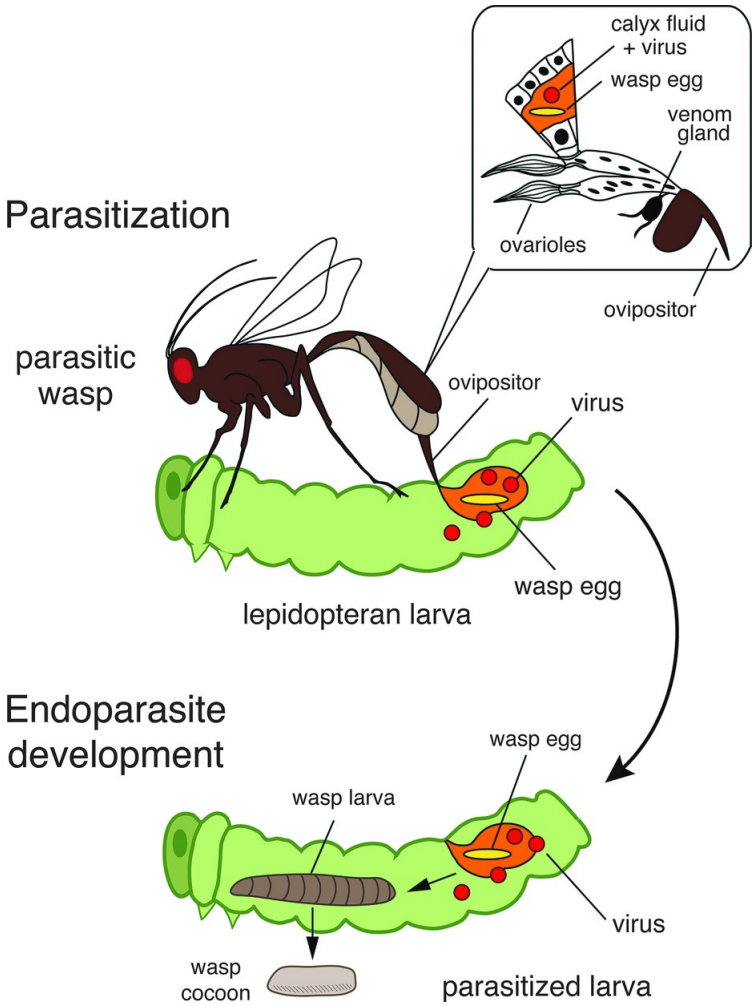

FIGURE 19.7 **Transmission of the polydnaviruses (*Polydnaviriformidae*)**. DNA-containing particles form in the epithelial cells of adult female ovaries and are released into the calyx fluid (**inset**) where they accumulate to high densities. In the first step of parasitization (*Top*), the wasp egg is injected along with virus and venom into a lepidopteran larva during oviposition. During endoparasitic development (*Bottom*), *Polydnaviriformidae*-encoded gene products suppress the larval immune response allowing the wasp egg to develop. After parasitization, the wasp larva spins a cocoon, pupates, and emerges as an adult to repeat the process. (From Dr. Bruce A. Webb, University of Kentucky; modified with permission.)

The family *Polydnaviridae* derived its name from the taxonomically characteristic "polydisperse" DNAs that comprise the multisegmented genome of these agents.[181,301,303] Polydnavirus particles contain numerous double-stranded, supercoiled DNA molecules with sizes ranging from 2 to >30 kbp. The particle's DNA is generated from the polydispersed proviral DNA embedded within the host wasp's genome. These genomic DNA fragments are amplified and packaged selectively as circular segments within the particle.[19,303] Remarkably, genome replication and particle assembly occur only in the wasp, not in the parasitized, immune-compromised caterpillar. Compelling metagenomic evidence suggests that the polydnaviruses are domesticated endogenous virus elements derived originally from the large DNA nudivirus family.[75,305] The close relationship between polydnaviruses and the nudiviruses was originally suggested by sequence similarities[18,336] between polydnavirus RNA transcripts expressed in the ovaries (the site of virus replication) of endoparasitic wasps with transcripts of nudivirus-infected insects. In contrast to the polydnaviruses, the nudiviruses have an autonomous life cycle, yet interestingly they also have the capacity to integrate into the host insect genome[257] (see below).

Polydnaviruses have been isolated from the *Ichneumonidae* and *Braconidae* families of wasps and are correspondingly divided into the ichnoviruses and bracoviruses, respectively.[19,181,303] The ichnovirus particles are ellipsoid with a uniform size of ~90 × 300 nm (Fig. 19.8); they resemble particles of the insect ascoviruses (see below). The nucleocapsid is enveloped by two-unit membranes, one derived from the nucleus during particle morphogenesis and the other by budding from the plasma membrane. In contrast, bracovirus particles consist of enveloped rod-shaped nucleocapsids with a uniform diameter (35-40 nm) and variable length (30-200 nm); they more closely resemble the nucleocapsids of nudiviruses and baculoviruses. Bracoviruses have a single membrane envelope, which is derived from the nucleus and surrounds one or more nucleocapsids (Fig. 19.8). Polydnavirus particles contain twenty or more virus-encoded polypeptides associated with the nucleocapsid and the membrane envelope. These structural proteins are produced in the wasp through the expression of proviral DNA.[19,181,303]

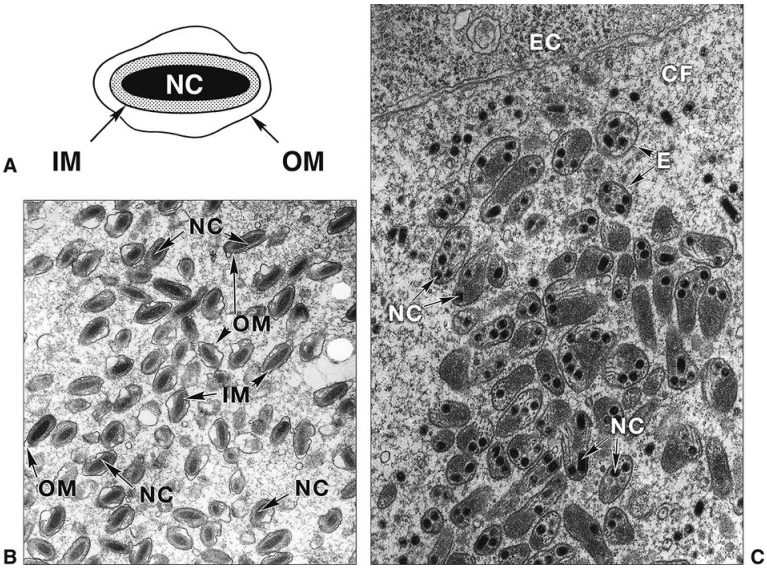

FIGURE 19.8 **Morphological features of the polydnaviruses (*Polydnaviriformidae*)**. **A**. Schematic of an ichnovirus particle. An inner (IM) and outer (OM) membrane surrounds the nucleocapsid (NC). **B**. Electron micrograph of an ichnovirus. Inner and outer membranes envelope the lenticular nucleocapsids (NC) of uniform size. **C**. Electron micrograph of bracovirus in the calyx fluid (CF) of a female parasitoid wasp. A single membrane envelope (E) surrounds multiple nucleocapsids (NC) viewed end-on or laterally. An epithelial cell (EC) of the calyx is shown. (Micrographs provided by Dr. Donald B. Stoltz, Dalhousie University. Modified from Stoltz DB, Vinson SB. Viruses and parasitism in insects. *Adv Virus Res*. 1979;24:125-171. Copyright © 1979 Elsevier, with permission.)

Polydnavirus Genome Organization

The polydnavirus genome is unique among animal viruses. It consists of an interrelated assortment of covalently closed, double-stranded DNA circles that exist in multiple topologies that include superhelical and relaxed circular forms. The complete genome sequence of many bracoviruses and ichnoviruses is known. The packaged DNAs (designated by letters) are nonequimolar and polymorphic in length.[181,301,303] For example, the 568-kbp genome of *Cotesia congregata* bracovirus (CcBV) consists of 30 dsDNAs ranging from 10 to >30 kbp.[79] Although each segment encodes at least one gene, a majority of the CcBV genome is comprised of noncoding DNA, in contrast to that of most autonomous DNA viruses. Not surprisingly, the aggregate size of polydnavirus genomes varies greatly and can be as large 600 kbp.[303] Many polydnavirus genes contain introns and thus resemble host genes. In the case of the ichnovirus prototype, *Campoletis sonorensis* polydnavirus (*Cs*IV), the encapsidated 247-kbp genome is comprised of 24 nonredundant circular DNA segments.[350] However, the *Cs*IV genome also contains multiple redundant segments and multiple repetitive elements. These higher copy number segments may provide increased expression of genes encoded therein. Each *Cs*IV genome segment encodes at least one gene but also contains portions that are homologous to other segments. Several of the ichnovirus segments are likely derived by recombination from larger segments and are thus described as "nested" segments.[181,303] This phenomenon also increases the copy number of certain virulence genes.

The packaged DNA of the bracoviruses and the ichnoviruses lack genes that are characteristic of known virus families, including those involved in DNA replication.[19,303] Instead, the particle DNAs encode several novel gene families that contribute to the pathology and immune modulation by these viruses in the parasitized host (see below). Thus, in the absence of any virus multiplication outside the wasp itself (ie, in the parasitized caterpillar), replicative genes are dispensable. Relevant to the origin of polydnaviruses, specifically the bracoviruses, is the finding that provirus genes expressed in the wasp ovaries include a set of 22 genes that are most closely related to those encoded by the autonomous nudiviruses that also infect lepidopteran caterpillars.[18] Of these bracovirus genes, 14 individual genes exhibit sequence similarity to those baculovirus genes involved in late gene transcription, virus DNA packaging, particle assembly, and membrane envelopment. However, there is no evidence for expression of those genes known to contribute to baculovirus DNA replication, including the *lefs* encoding DNA helicase, DNA polymerase, or DNA binding proteins. These findings and others support the notion that these polydnaviruses evolved from an ancestral nudivirus infection in the braconid wasps ~100 million years ago.[19,303,305] Sequence analyses of nonencapsidated ichnovirus structural genes expressed in ovaries of ichneumonid wasps suggest that the ichnoviruses may also have evolved from a yet to be identified ancestral virus unrelated to the bracoviruses.[305,336]

Proviral DNA of the polydnaviruses is present as integrated segments that are clustered at different loci of the wasp genome; thus, the provirus is inherited in a mendelian fashion. The mechanisms by which the polydnavirus genome is amplified, excised, and packaged into virus particles are poorly understood.[181,303] Evidence exists that proviral DNA segments within the wasp genome are amplified before excision and encapsulation. Where the relationship between the particle's DNA and the proviral DNA is known, the largest or "master" DNA segments within the wasp genome are flanked by direct repeats. For example, the 16-kbp integrated W segment of *Cs*IV is flanked by 1.2-kb repeats.[67,181] Within the W segment, additional 350-bp repeats generate nested segments R and M, apparently by homologous recombination during amplification in the female wasp. In the wasp genome, *Cs*IV segment B is flanked by 59-bp direct repeats that are removed during excision and circularization.[88] This finding and others are consistent with a mechanism in which *Cs*IV proviral segments are excised from the wasp genome, possibly by recombination, prior to amplification.[118] The presence of chromosomal sites lacking proviral DNA in tissues that accommodate viral replication is consistent with this later mechanism.

Polydnavirus Contributions to the Host's Life Cycle

Polydnaviruses play a critical role in the life cycle of endoparasitic wasps and contribute to their reproductive success. These endogenous viruses

multiply in the oviducts of female wasps as a normal step in the development of the reproductive tract (Fig. 19.7). Virus multiplication is under hormonal control and initiates during development of the wasp as a pupa.[181,303] Virus DNA production, encapsidation, and particle assembly occurs in the nucleus of epithelial calyx cells of the wasp's oviducts (Fig. 19.7). In mature females, the particles are released from the calyx cells into the oviduct lumen, either by budding (ichnoviruses) or by cell lysis (bracoviruses). Polydnavirus particles accumulate to high density in the calyx fluid and are deposited along with the egg and other wasp secretions in the target larva during oviposition (Fig. 19.7). Virus gene expression in the wasp differs from that in parasitized caterpillar hosts.[181,303] Despite the presence of high concentrations of particles, polydnaviruses cause no obvious pathology in provirus-carrying wasps. The predominant form of viral DNA in the wasp male is the integrated provirus.

Polydnavirus Modulation of Larval Responses During Parasitization

Upon oviposition, the female wasp delivers her egg accompanied by virus particles to the recipient caterpillar (Fig. 19.7). The caterpillar normally mounts an immune response that encapsulates and destroys the intruding egg in a process mediated in part by hemocytes of the larva's circulatory system. However, in the case of polydnavirus-carrying ichneumonid and braconid wasps, the virus suppresses encapsulation and other aspects of the larva's immune response, which allows proper development of the wasp's offspring. In experiments where the egg is artificially injected into the larva in the absence of virus, the egg is destroyed, thereby demonstrating a definitive role for virus-mediated abrogation of the larva's immune response.[78] Thus, gene expression of the delivered virus particles function to protect the wasp egg from the immune response of the recipient larva.

The most obvious physiological effect of the polydnaviruses is in the parasitized insect host, where larval immunity and development are altered.[181,303] Neither virus replication nor virus assembly occurs in parasitized larva. However, particle DNA is long-lived and viral RNA transcription occurs throughout parasitization, principally in larval hemocytes and fatbody (the equivalent of the vertebrate liver). In caterpillars, the primary immune response to parasitization is encapsulation of the wasp egg and includes direct attachment of larval hemocytes in layers. Polydnaviruses suppress encapsulation by altering the physiology of the capsule-forming hemocytes (granulocytes and plasmatocytes).[303] Some polydnavirus gene products are secreted and may affect surrounding hemocytes and tissues. In the case of *Microplitis demolitor* bracovirus (MdBV), virus-induced apoptosis kills a subclass of hemocytes that mediate the encapsidation response.[306] Nonetheless, some hemocytes survive to express polydnavirus genes that alter their function.

Polydnavirus particles carry the genes for suppression of the caterpillar's immune response.[181,303] Multiple gene families contribute to immune modulation. This repetition and variation of related pathogenicity genes may enhance expression levels or facilitate tissue targeting during parasitization. One such gene family expressed during parasitization includes the viral ankyrin (*vankyrin*) genes, which encode factors with ankyrin repeat domains belonging to the Iκβ family of proteins.[182,322] Two *vankyrin* genes of MdBV exhibit normal Iκβ activity by inhibiting NF-κβ responsive genes when exogenously expressed in *Drosophila* cells.[322] Suggestive of their importance in immune modulation, *vankyrin* genes are encoded by both the ichnoviruses and the bracoviruses. NF-κβ is an important regulator of insect genes encoding antimicrobial peptides and factors involved in cellular immunity.[303] Thus, the Iκβ-like activity of the *vankyrin*s could disrupt NF-κβ activation of these immune response genes in the parasitized larva. Polydnaviruses also carry a family of genes (*ptp*) that encode tyrosine phosphatase–like proteins (PTPs). Cellular PTPs regulate receptor–mediated signal transduction pathways, suggesting that the virus-encoded PTPs function similarly to downregulate the immune response. Indeed, two bracovirus *ptp*s are involved in suppression of encapsulation and phagocytosis.[264,307] Polydnavirus pathogenesis genes can also have either proapoptotic or antiapoptotic effects on lepidopteran cells,[80,307] suggesting that virus-mediated control of apoptosis contributes to immune suppression. Other polydnavirus-encoded immune modulator genes, including those predicted to encode epidermal growth factor–related proteins, cysteine-rich motif proteins, vinnexin, and *rep* proteins, have been identified.[79,181,303]

The *Polydnaviridae* family is a fascinating group of agents that challenge the definition of a virus. On the basis that the polydnaviruses represent a type of mobile genetic element, incapable of autonomous replication, but which evolved from host wasp genes and virus capsid genes in order to contribute positively to the wasp's life cycle (see above), the ICTV has recognized these entities as a category distinct from viruses, designating them "viriforms" (*Polydnaviriformidae*). On the other hand, persuasive evidence has suggested that the polydnaviruses are a type of "domesticated" endogenous virus.[75,305] The multiple proliferative genes conserved between the bracoviruses and the autonomous-replicating insect nudiviruses, as well the abundance of functional virus particles, suggest that the "polydna" bracoviruses were derived through genomic integration of an ancient nudivirus, which was followed by evolutionary loss of autonomy. Likewise, the nonautonomous ichnoviruses may have evolved from a yet to be identified ancestral virus, which was capable of genomic integration. Continued study of *Polydnaviridae* or *Polydnaviriformidae* will undoubtedly provide key insight into the evolution of these novel virus elements and promises to reveal fundamental understanding of the insect immune system and its regulation.

Ascoviridae

Classification and Structure of Ascoviruses

Ascoviridae is a family of large DNA viruses that primarily infect insects of the order Lepidoptera (moths and butterflies) and cause a slow fatal disease.[84,85,355] The name ascovirus is derived from the Greek word *askós* (sac), which describes the large membrane-bound vesicles that are associated with virus development. The formation of these virion-containing vesicles represents the family's distinguishing feature[81,82,85] and is critical to the virus' dissemination strategy that involves endoparasitic wasps (see below). The ascoviruses are one of the newest DNA virus families to be discovered, with a limited number of species identified to date. Depending on the species, ascovirus particles are reniform (kidneylike) or bacilliform in shape (Fig. 19.9A). The virions (~400 × 130 nm) have an electron dense core surrounded by two-unit membrane envelopes (Fig. 19.9B). At least 21 different proteins, ranging in size from 9 to 120 kDa, are present in virions of the prototype ascovirus, *Spodoptera frugiperda* ascovirus 1a (SfAV).[312] The ascovirus genome is a double-stranded circle of DNA ranging in size from ~150 to 190 kbp, depending on the species and encodes from 120 to 180 open reading frames.[84,355] Nucleotide sequence analyses indicate that the ascovirus family is unique but exhibits some relatedness to the invertebrate iridoviruses.[21,355]

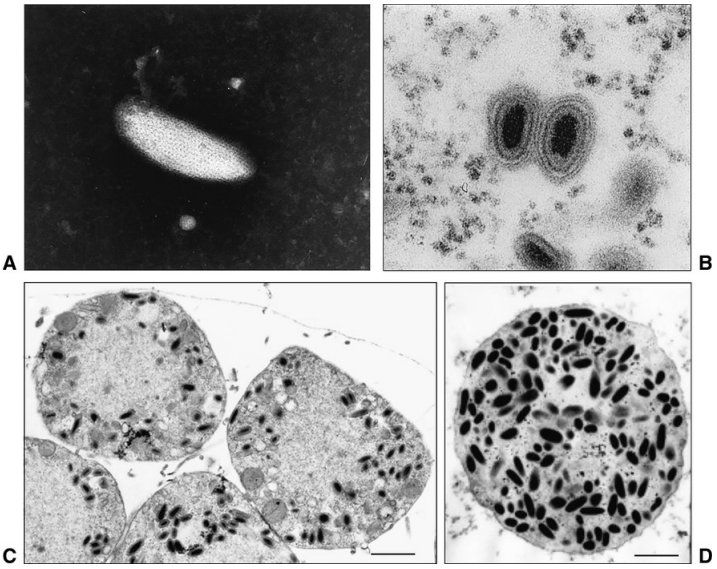

FIGURE 19.9 **Ascoviruses**. **A.**A single virion of *Trichoplusia ni ascovirus* (TnAV). The kidney-shaped virion has a reticular surface when negatively stained and examined by electron microscopy. **B.** Cross section of two ascoviruses. Virions in the larval fat body of *Scottogramma trifolii* (Lepidoptera, family Noctuidae) are imaged through the middle of their long axis. A multilayered membrane surrounds the central nucleocapsid. **C.** Intracellular vesicles formed from a TnAV-infected fat body cell. The electron micrograph shows four nascent viral vesicles bordered by the fat body's basement membrane (*thin line* at the *top*) that has not yet ruptured to release these vesicles into the hemolymph. Both mature (enveloped) and immature (nonenveloped) TnAV nucleocapsids (dark stained, kidney shapes) are present. **D.** A mature TnAV viral vesicle circulating in the hemolymph. The grazing peripheral electron micrograph shows mostly mature virions ready to be released into the hemolymph. Bars: 800 nm. (**A** courtesy of Dr. Brian A. Federici, University of California-Riverside and Dr. J. J. Hamm, USDA-ARS, Tifton, GA; **B** modified from Federici BA. A new type of insect pathogen in larvae of the Clover cutworm Scotogramma trifolii. *J Invertebr Pathol*. 1982;40(1):41-54. Copyright © 1982 Elsevier, with permission. **C and D** reprinted from Zaghloul HAH, Hice RH, Arensburger P, et al. Extended in vivo transcriptomes of two ascoviruses with different tissue tropisms reveal alternative mechanisms for enhancing virus reproduction in hemolymph. *Sci Rep*. 2021;11(1):16402. http://creativecommons.org/licenses/by/4.0/.

Stages of Ascovirus Replication

The first visible sign of ascovirus infection is hypertrophy of the cell and its nucleus; the infected cell's diameter can increase up to 10-fold.[83,85] The nucleus subsequently fragments and the infected cell is divided into 20-30 membrane-bound vesicles containing assembling virions (Fig. 19.9C). This process involves extensive invagination of the plasma membrane and mimics that which occurs during apoptosis. Once virus-containing vesicles dissociate from the infected cell, they are released into the larva's hemolymph. The larva's basement membranes are then disrupted and the hemocoel fills with ~10^8 vesicles/mL, which gives the hemolymph a distinguishing milky white color.[81,82] These vesicles with their associated virus are subsequently picked up during the egg laying process by the parasitic wasp's ovipositor.

Interestingly, a hostlike effector caspase encoded by the ascovirus SfAV likely contributes to this unusual vesicle formation during infection.[20] Thus, the ascoviruses may have exploited host cell apoptosis to expedite virus transmission by endoparasitic wasps (see below). *Heliothis virescens* ascovirus (HvAV) also encodes a caspase, but multiplication in cell lines fails to induce comparable vesicle formation.[2] Thus, the role of ascovirus caspases or that of the host's caspases during larval infection remains to be clarified. The ascoviruses encode several lipid metabolism enzymes, which may also contribute to vesicle production.[84]

To date, ascovirus replication mechanisms are poorly understood. RNA-sequencing studies that surveyed the SfAV transcriptome in infected caterpillars revealed that transcription occurs predominantly in virus-generated vesicles, not in the originally infected tissues consisting mostly of the fat body.[370,371] This finding suggests that the assembly of infectious virions also occurs in the virus-generated vesicles. Thus, the ascovirus vesicle may represent a viral factory that is unique among DNA viruses.[371] Multiplication in lepidopteran cell cultures has revealed possible roles for viral and cellular microRNAs in regulation of *Heliothis virescens* HvAV.[4] Interestingly, HvAV and other ascoviruses encode an RNAse III gene, which exhibits nuclease activity for double-stranded RNA and appears to be essential for virus multiplication.[153] Encoded by few other viruses, RNAse III proteins play an important role in RNA silencing.

Ascovirus Transmission, Disease, and Host Range

In nature, the principal transmission route of ascoviruses involves endoparasitic wasps, which carry virus-containing vesicles on their ovipositor as a result of egg laying. In the laboratory, ascovirus inoculation is accomplished mechanically by pricking the host larva with a needle containing virus-containing vesicles.[84] A dose of 10 larva-derived vesicles is sufficient for 90% infection. The viruses cause a slow, chronic infection in larvae or pupae. The disease causes reduced feeding, developmental arrest, and death within 2-6 weeks. There is little obvious pathology during the early stages of infection. Some larvae exhibit the characteristic milky discoloration due to the abundance of virus-containing vesicles. The host range of ascoviruses may include only a few closely related insect species, mostly of the order Lepidoptera.[84] However, the lack of obvious pathology early in infection may mean that the host range is broader than that suggested by entomological surveys.

Nudiviruses

Classification, Structure, and Genomics of the Nudiviruses

Historically, the nudiviruses were thought to be nonoccluded baculoviruses because of their common characteristics, including rod-shaped nucleocapsids, a circular, double-stranded DNA genome, and nuclear replication.[37] However, nucleotide sequence analyses subsequently revealed that the nudiviruses are only distantly related to the baculoviruses.[257,347,355] Thus, the new family *Nudiviridae* accommodates these novel insect pathogens. The nonoccluded nature of the virion confers the virus family name "nudi" from the Latin word "*nudus*" (naked). Nudiviruses infect multiple orders of insects, including the Diptera, Lepidoptera, Coleoptera, and Orthoptera, as well as some aquatic crustaceans. They are divided into four genera: the alpha-, beta-, delta-, and gamma-nudiviruses.[136,257]

Varying in size from 97 to 232 kbp, the nudivirus DNA genome is packaged into a nucleocapsid that is surrounded by a membrane envelope acquired within the nucleus and one obtained upon budding from the plasma membrane. As determined from nucleotide sequence analyses, there are 32 core genes present in all nudivirus genomes.[136,257] Of these conserved core genes, 20 exhibit sequence similarity with baculovirus and bracovirus genes that are involved in transcription, DNA replication, and nucleocapsid structure or assembly.[257,347] In general, nudivirus replication mechanisms are poorly understood, so the exact functions of these nudivirus core genes are unknown. Infection can cause cell lysis and insect mortality or establish a persistent or latent infection (see below). Although characterized as nonoccluded viruses, certain nudiviruses also possess a gene related to polyhedrin or granulin used by baculoviruses for occlusion. It is interesting to speculate that this occlusionlike gene is the remnant of a common ancestor of the nudiviruses and baculoviruses.

Nudivirus Latency and Transmission

Originally isolated from a *Heliothis zea* (corn earworm) cell line, HzV-1 is one of the best-characterized nudiviruses (genus *Betanudiviridae*). The HzV-1 genome is a large (~228 kbp) circle of double-stranded DNA[52] that possesses ~154 ORFs, many of which have striking sequence similarity to the core baculovirus replication genes. A hallmark of HzV-1 is its capacity to establish persistent or latent infections.[37] HzV-1 persistence involves stable integration of the genomic DNA and low-level, spontaneous replication of virus that can cause lytic infection of naive cells.[197] The establishment of viral persistence or latency is associated with the presence of a single 2.9-kb HzV-1 RNA transcript, designated the persistency-associated transcript (PAT1).[47] PAT1 is a noncoding RNA that is the only HzV-1 RNA detected during latency. PAT1 appears to suppress the HzV-1 *hhi*-1 gene by a mechanism that involves the generation of microRNAs that target and destabilize *hhi*-1 RNA transcripts.[363] Transcription of HzV-1 *hhi*-1 gene occurs early in infection and mediates reactivation from latency, thereby contributing to a productive infection.[362] Consequently, HzV-1 may use microRNAs derived from its single latency transcript in an RNA interference mechanism that is required for establishment of latency.[363] Thus, HzV-1 will serve as a useful model for mechanistic studies of insect virus latency.

Oryctes virus is another notable nudivirus (genus *Alphanudivirus*). Distinguished by its unique tail-like structure protruding from one end, this 128-kb DNA virus shares many genes with other nudiviruses and certain replication, transcription, and nucleocapsid genes with the baculoviruses.[346] Oryctes virus causes fatal infections of the rhinoceros beetle *Oryctes rhinoceros*, which is a serious pest species of palm trees in South-East Asia and the Pacific region.[156] Consequently, this virus is a very effective biological control agent used in coconut and palm oil plantations of the area.[250] Virus is transmitted by contaminated food sources or mating. Typical of most nudiviruses, little is known about its replication mechanisms.

Nodaviridae

Classification and Host Range of Nodaviruses

Members of the family *Nodaviridae* are small RNA viruses with a single-stranded, positive-sense genome consisting of two RNAs. The bipartite genome is packaged within a single, nonenveloped virion (~30 nm dia) with T = 3 icosahedral symmetry.[9,11,333] The genome organization and replication strategy of the nodaviruses is among the simplest of known viruses (Fig. 19.10). As such, they are powerful models for exploring strategies of virus RNA replication, virion assembly, host interactions, and RNA silencing.

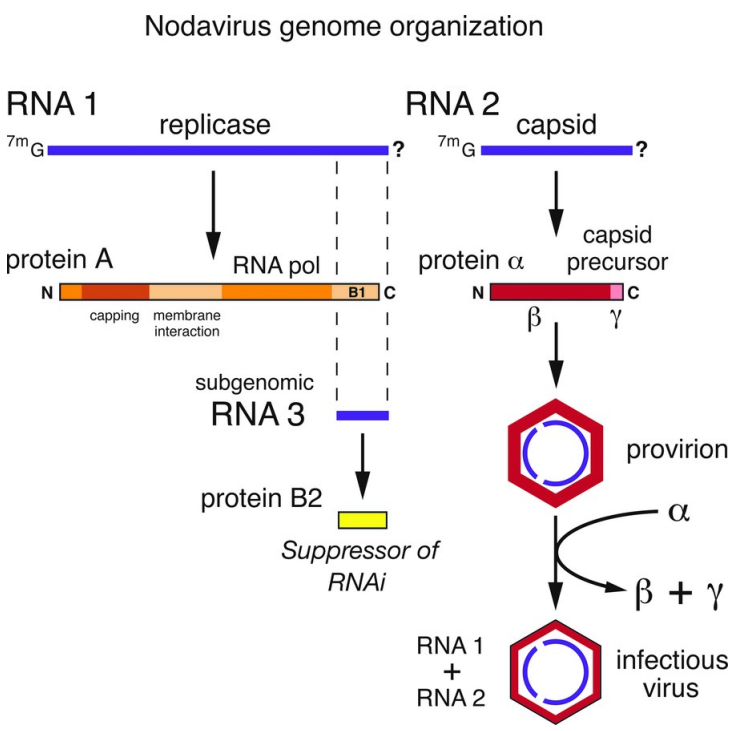

FIGURE 19.10 **Nodavirus genome organization and assembly**. Positive-sense genomic RNAs 1 and 2 encode the replicase (protein A) and capsid precursor protein (protein α), respectively. Protein A possesses RNA capping, membrane interaction, and RdRp domains. Derived from RNA 1, subgenomic RNA 3 encodes protein B2, which functions as a suppressor of host cell RNA silencing (RNAi). Assembled from capsid precursor α, nodavirus provirions undergo a required maturation cleavage, which yields capsid proteins β and γ. Infectious virions contain an equimolar ratio of genomic RNA 1 and 2.

The nodaviruses are divided into two genera, the alphanodaviruses and betanodaviruses, which infect insects and fish, respectively. Distinct from alpha- and betanodaviruses, another group of nodaviruses associated with crustaceans, including shrimp or pawn, have been recently identified and tentatively classified as gammanodaviruses.[277] The Nodavirus family name originated from Nodamura virus, which was first discovered in mosquitoes near the Japanese village of Nodamura.[282] The best-studied insect nodaviruses include flock house virus (FHV), black beetle virus (BBV), Nodamura virus (NoV), and Wuhan nodavirus (WhNV). These viruses were isolated from the grass grub *Costelytra zealandica* (Coleoptera) in New Zealand, the scarab beetle *Heteronychus arator* (Coleoptera) also in New Zealand, the mosquito *Culex tritaeniorhynchus* (Diptera) in Japan, and the Small Cabbage White butterfly *Pieris rapae* (Lepidoptera) in China, respectively.[333] Most members of the alphanodavirus genus were isolated from insects of Australasia. Other alphanodaviruses include Pariacoto virus (PaV), New Zealand Virus, Boolarra virus, and Manawatu virus. NoV is the only nodavirus with the capacity to multiply in vertebrates.[9] Nonetheless, upon transduction of their genomic RNA, many nodaviruses can replicate in cells from highly diverse organisms, including mammals, nematodes, plants, and the yeast *Saccharomyces cerevisae*.[6,204,260,287]

Nodavirus Virion Structure

The nodaviruses have provided enormous insight into icosahedral virus assembly and maturation. The structures of BBV, FHV, NoV, PaV, and others have been determined at atomic resolution (go to: http://bcrf.biochem.wisc.edu or http://viperdb.scripps.edu/).[11,87,148,313,375] Each virion contains 180 copies of the same capsid protein (Fig. 19.11A). Sixty triangular units, consisting of three similarly folded capsid proteins, form an icosahedral lattice with T = 3 symmetry.[158,283] Three capsid proteins form a prominent peak at the quasi-threefold axis. At the twofold axes, conserved C-terminal residues of the capsid protein make contact with duplexed genomic RNA that is highly ordered and stabilize the capsid protein interface.[87] The structures of nodaviruses, including that of PaV and FHV, reveals a dodecahedral cage of ordered double-stranded RNA (Fig. 19.11B) that constitutes 35% (1500 nucleotides) of the single-stranded RNA genome.[313,323] The RNAs interact extensively with the basic N-terminal region of the capsid protein. C-terminal residues of the capsid protein are required for RNA encapsulation.[333] At the fivefold axis, the capsid proteins form a pentameric helical bundle that has been proposed to participate in release of the genomic RNA upon virus penetration into the host cell's cytosol.[53]

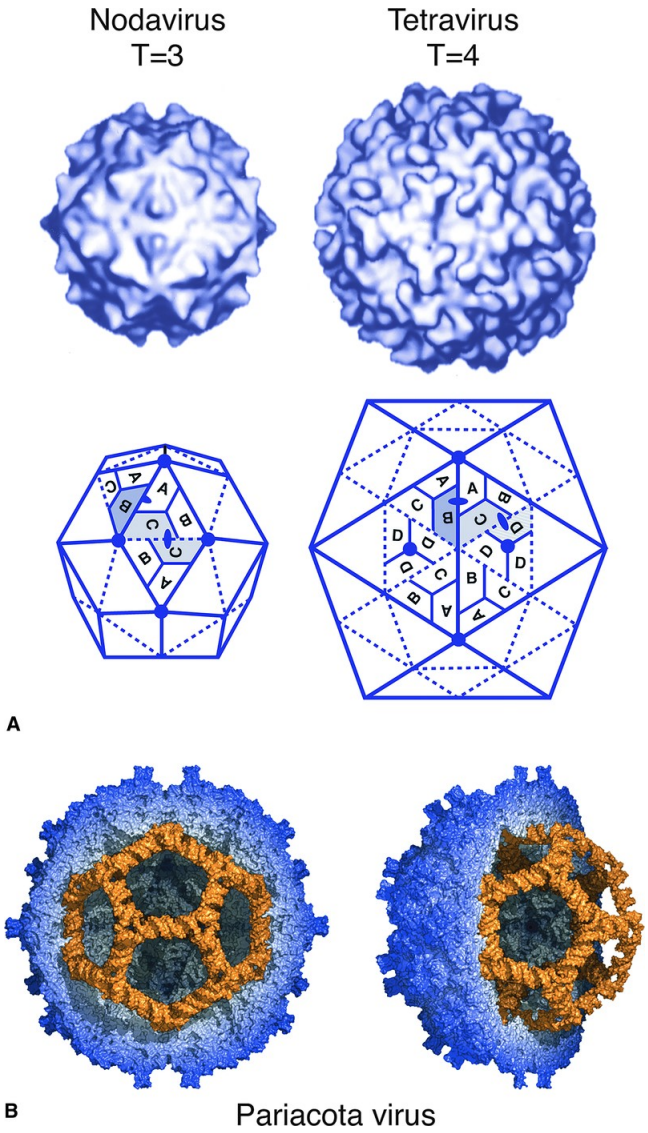

FIGURE 19.11 **Comparison of nodavirus and tetravirus structures**. **A**.(*Top*) Image reconstructions of a T = 3 nodavirus (flock house virus) and a T = 4 tetravirus (*Nudaurelia capensis* ω virus). The average particle diameter for the nodaviruses and tetraviruses is 320 and 415 Å, respectively. (*Bottom*) Subunit arrangement of the nodavirus (T = 3) and the tetravirus (T = 4) virions. Each subunit is indicated by a trapezoid. Nodaviruses contain 60 subunits of three similarly folded capsid proteins (A, B, and C) that form a prominent peak at the quasi-threefold axis (*top* image). Tetraviruses contain 240 capsid protein subunits (A, B, C, and D) in which 12 subunits comprise each triangular face of the virion (see images at http://bcrf.biochem.wisc.edu or http://viperdb.scripps.edu/). **B**. Arrangement of genomic RNAs within the virion of the nodavirus Pariacoto virus. The symmetrically arranged shell of helical RNA (*orange*) contacts capsid proteins on the inside surface of the virion (*blue*). (Adapted with permission from Dr. John E. Johnson, Scripps Research Institute, La Jolla, CA. Images of Pariacoto virus were kindly provided by Jean-Yves Sgro, Department of Biochemistry, University of Wisconsin-Madison.)

The nodavirus provirion is assembled from 180 copies of the ~43-kDa capsid precursor α. During virion maturation (Fig. 19.10), α is cleaved very near its C terminus to generate capsid proteins β and γ.[96,103] For FHV, this autocatalytic postassembly cleavage occurs between residues Asn^{363} and Ala^{364} which are located on the inside surface of the virion. This cleavage event is required for infectivity and virion stability.[103,284]

Nodavirus Genome Organization

The nodavirus genome consists of two single-stranded, messenger-active RNAs, designated RNA 1 and 2. Both RNAs are 7-methyl GpppN capped but not polyadenylated (Fig. 19.10). The 3′ ends of both RNAs have an unknown structure or unidentified factor attached. RNA 1 (~3.1 kb) encodes the functions required for RNA transcription and replication, whereas RNA 2 (~1.4 kb) encodes the single coat protein α. Both RNAs are required for infectivity and are packaged within the same virion.[333] RNA 1 carries all the information necessary for autonomous replication.[102] It encodes at least three polypeptides: proteins A, B1, and B2 (Fig. 19.10). Protein A (~112 kDa) is an RNA-dependent RNA polymerase (RdRp) that is required for virus RNA replication.[8,102] Encoded by the 3′ end of RNA 1, protein B1 has an unknown function. Protein B2 (~12 kDa) is translated from subgenomic RNA 3 (Fig. 19.10), which is derived from the 3′ end of RNA 1. In infected cells, RNA 3 is abundant but not packaged into virions.[97,120] Protein B2 is a suppressor of host-mediated RNA silencing (see below). Nodavirus genomic RNA 2 encodes the capsid precursor protein α, which is autocatalytically cleaved to yield capsid protein β and peptide γ (Fig. 19.10) during maturation. Thus, the functions necessary for genome replication (RNA 1) and virus transmission (RNA 2) are segregated onto different genetic elements.

Stages of Nodavirus Replication

As the best-studied nodavirus, FHV multiplies prolifically in *Drosophila melanogaster* cell lines.[333] This system has proven highly tractable for molecular studies of virus replication mechanisms. Although the *Drosophila* receptor is unknown, it is likely that FHV enters by receptor-mediated endocytosis. The endosome's acidity triggers an irreversible exposure or release of the capsid γ peptides, which disrupts the endosomal membrane and facilitates release of the genomic RNAs into the cytoplasm.[340] Required for membrane penetration, the lipophilic γ peptide is generated by autoproteolytic cleavage of capsid protein precursor α (Fig. 19.10) within the assembled virion.[10,232]

Immediately upon release in the cytoplasm, translation of the positive-sense genomic RNAs begins. RNA 1 produces protein A, the RdRp that carries RNA capping activity and membrane-association domains. Protein A interacts with the outer mitochondrial membrane to form spherical (~50-nm diameter) lipid vesicles called spherules[215] (Fig. 19.12A and 12B). Both protein A and replicating FHV RNA are required for spherule formation.[171] Mitochondrial phospholipids and cellular chaperones are critical for FHV RNA replication and thus are also likely important for spherule assembly.[299,352] A single replicating RNA intermediate, located inside the spherule, is connected to the outside cytoplasm by a single necklike portal rimmed with protein A (Fig. 19.12C). CryoEM studies[326] have indicated that protein A oligomerizes to form a 12-fold symmetrical crown structure on the cytosolic side of the membrane portal (Fig. 19.12D). The striking crown contains 12-fold symmetrical projections that rim the portal's entrance/exit. It is thought that the protein A–containing crown assembly plays a critical role in viral RNA production as protein A has both RdRp and RNA-capping functions.[326] Remaining to be explained is how protein A recruits the initiating positive-sense RNA template to generate the spherule with negative-sense replicative intermediate RNA on the inside that ultimately yields high levels of positive-sense RNA for translation and packaging.[326] Newly synthesized nodavirus RNAs are detected within 2 hours after inoculation and continue to accumulate through 20 hours. By then, viral RNA represents up to 50% of the total cellular RNA, an astounding level considering that it is comparable to that of ribosomal RNAs.[96,98,103] It is noteworthy that many RNA viruses replicate their RNA in association with membranes and some in spherulelike vesicles, including the alphaviruses and distantly related coronaviruses. The nodaviruses offer many experimental advantages in probing the mechanistic details of this conserved replication process (Fig. 19.12).

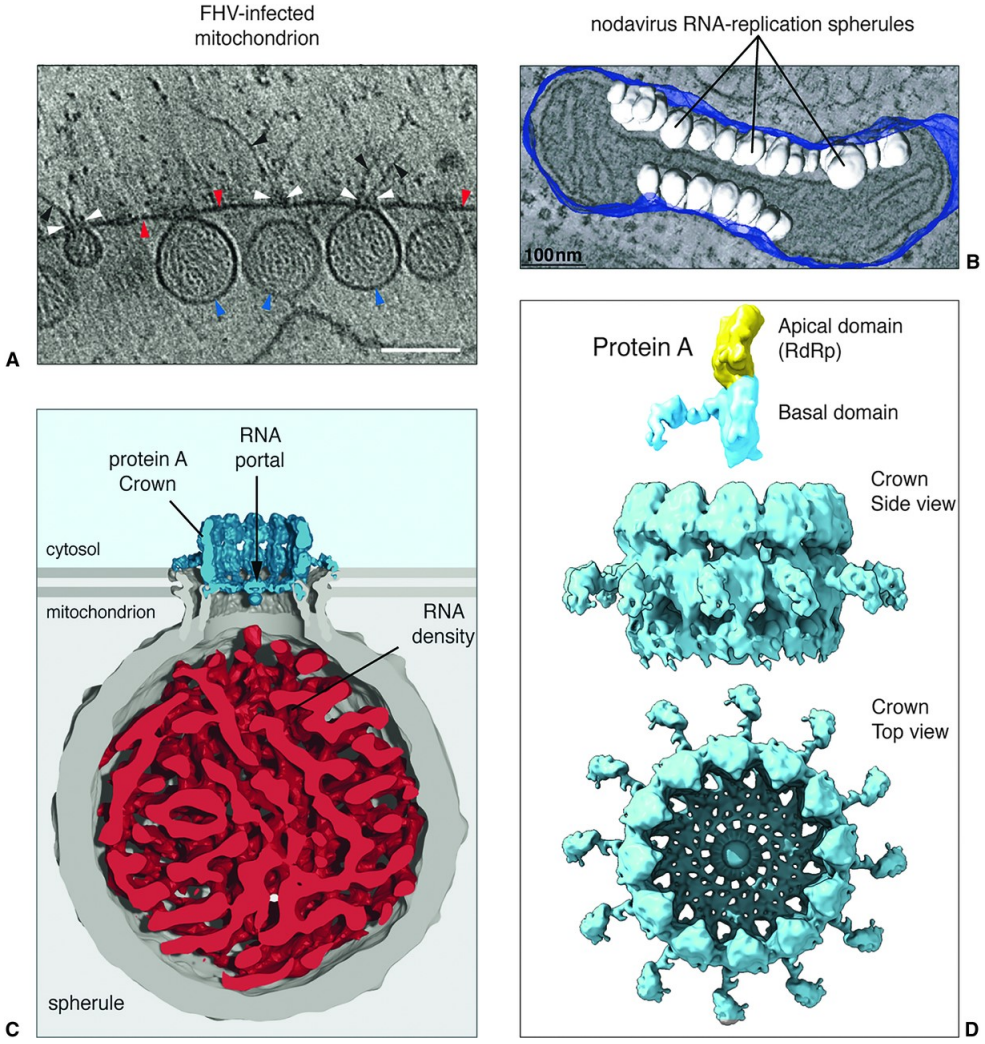

FIGURE 19.12 **Nodavirus RNA replication spherules and complexes**. **A**. Flock House virus–generated RNA replication spherules. An electron micrograph shows individual replication spherules (*blue arrows*) located on the inner mitochondrial membrane (*red arrows*) of an FHV-infected *Drosophila* cell. Putative viral RNA strands (*black arrows*) extend from the FHV protein A–containing crown (*white arrows*) positioned at the portal near the top of the spherule. (Ertel KJ, Benefield D, Castaño-Díez D, et al. Cryo-electron tomography reveals novel features of a viral RNA replication compartment. Elife. 2017;6:6:e25940. doi: 10.7554/eLife.25940) **B**. Mitochondrial image from an FHV-infected *Drosophila* cell. The three-dimensional tomographic reconstruction shows the mitochondrial membrane (*blue*) decorated with numerous RNA replication spherules (*white*). **C**. FHV replication spherule imaged by cryoEM. A reconstruction shows the replication spherule with RNA density (*red*) and the protein A crown (*blue*) positioned at the portal (*top*), which is embedded

in the mitochondrial membrane. **D**. High-resolution (8.5 Å) views of FHV protein A. A single protein A monomer (*top*) is shown with its globular domains and the membrane-anchoring extension. The side view (*middle*) and the top view (*bottom*) of the 12-fold symmetrical crown composed of protein A is shown as embedded within the membrane. (Courtesy of Paul Ahlquist, University of Wisconsin-Madison. Panel **A** taken from Ertel KJ, Benefield D, Castaño-Diez D, et al. Cryo-electron tomography reveals novel features of a viral RNA replication compartment. *Elife*. 2017;6:e25940. https://creativecommons.org/licenses/by/4.0/. Panels **B-D** adapted from Unchwaniwala N, Zhan H, den Boon JA, et al. Cryo-electron microscopy of nodavirus RNA replication organelles illuminates positive-strand RNA virus genome replication. Curr Opin Virol. 2021;51:74-79.)

During infection, nodavirus RNA synthesis is temporally regulated. Late in infection, the replication of genomic RNA 1 and 2 is coupled, which ensures production of an equal molar ratio of both RNAs required for virion packaging. However, early in infection, subgenomic RNA3, which produces B2 to suppress the host RNAi response, transactivates RNA2 replication.[7] As the level of RNA2 increases, it represses RNA3 production[102] through a feedback inhibitory mechanism.[333] *Cis*-acting sequences required for (+) and (−) strand RNA transcription and RNA 3 suppression have been identified.[9] The FHV RNA 1 nucleotide sequences that direct self-replication and RNA 1 recruitment to the mitochondrial membranes have also been defined.[328]

Translation of nodavirus RNA 2 yields abundant levels of the capsid precursor α, which is cleaved during capsid assembly (Fig. 19.10). Despite equal levels of intracellular RNA 1 and 2, synthesis of capsid protein α predominates late in infection as a direct result of the ability of RNA 2 to out compete RNA 1 for rate-limiting translation factors.[98] As capsid precursor α accumulates, an equimolar ratio of RNA 1 and 2 are packaged within the provirion; the mechanism that ensures this 1:1 stoichiometry is unknown. Before cell lysis, extraordinary yields of assembled particles (2 mg per 10^8 cells) accumulate in the cytosol of the infected cell and extensive crystalline arrays in the cytoplasm are often observed. Owing to the segmentation of the genome, reassortment of genomic RNAs occurs in mixed infections. Defective interfering particles appear often and can establish persistent nodavirus infections in cell culture.[162,333]

Permissive *Drosophila* cells respond to nodavirus FHV infection by undergoing caspase-dependent apoptosis.[290] Indeed, both alphanodaviruses and betanodaviruses can trigger apoptosis during multiplication.[129,290] FHV-mediated depletion of the endogenous inhibitor of apoptosis DIAP1 triggers apoptosis in *Drosophila*, as it does during baculovirus-induced apoptosis in these cells.[290,332] Because host caspases are not required for nodavirus assembly or maturation, the process of apoptosis and the resulting membrane-protected vesicles may facilitate virus release and subsequent dissemination to tissues within the infected host insect.

Nodavirus Suppression of Host RNA Silencing

Nodaviruses have also provided important understanding of the molecular mechanisms by which RNA viruses trigger the antiviral RNA silencing response in host cells and how viruses suppress the response through the activity of virus-encoded RNA-binding proteins.[1,72,333] RNA silencing or RNA interference (RNAi) is a critically effective antiviral defense in invertebrates.[30] Capable of targeting and destroying RNAs from viruses and transposons, RNAi may be a primordial form of innate immunity.[70] Nodavirus protein B2 functions to suppress RNA silencing that is mounted by the host cell in response to virus RNA replicative intermediates.[192] B2 of FHV was the first discovered example of an RNAi suppressor from an animal virus. B2 enhances FHV multiplication and virus-mediated killing in cultured cells as well as adult *Drosophila* flies.[101,159,192,345] Demonstrating the effectiveness of the RNAi response in insects, mutant flies with defective RNAi components are more susceptible to killing by FHV.[101,345] Interestingly, Nodamura virus B2, which grows in both vertebrates and invertebrates, can suppress the RNAi response in both.[192,194]

The anti-RNAi function of nodavirus B2 is attributed to its sequence-independent dsRNA binding activity.[1,333] B2 binds to dsRNA (probably dsRNA replicative intermediates generated during virus RNA synthesis) and prevents cleavage by the RNAaseIII-like endonucleases (Dicer) of the RNAi pathway. Thus, B2 suppresses the generation of small interfering RNAs that are complementary to viral RNAs and used to degrade viral messenger RNA. The crystal structure of FHV B2 in complex with dsRNA shows B2 as a small four-helix polypeptide that binds as a dimer to a single face of duplex RNA in a sequence-independent manner.[46] Nodamura virus B2 has a similar protein fold despite its divergent sequence.[172] It is likely that multiple copies of nodavirus B2 bind to viral dsRNAs, thereby protecting them from cleavage by Dicer and blocking incorporation into the RNAi silencing complexes.[46,267] It will be of interest to determine where B2's dsRNA binding activity occurs in the infected cell relative to the nodavirus RNA-replicating spherules on the mitochondrial membranes.

Tetraviridae

Classification of Tetraviruses

The tetraviruses are small RNA viruses that multiply almost exclusively in lepidopteran insects (moths and butterflies). They contain a single-stranded, positive-sense RNA genome that is packaged into a nonenveloped, icosahedral virion. The family name *Tetraviridae* ("tetra" from Greek *tettares*, meaning "four") is derived from the virion's T = 4 symmetry, which is the only known T = 4 symmetry among nonenveloped viruses.[73,74,158] A major contribution of these viruses has been their use as models for defining molecular mechanisms of assembly and maturation of icosahedral viruses. Recently, cell culture systems have been identified—thus, more information on tetravirus replication strategies will be available. Tetraviruses are represented by three families: *Alphatetraviridae*, *Permutotetraviridae*, and *Carmotetraviridae*. *Alphatetraviridae* is divided into two genera, *Omegatetravirus* and *Betatetravirus*, in which *Nudaurelia capensis* β virus (NβV) and *Nudaurelia capensis* ω virus (NωV) are the prototype species, respectively.[73] The tetravirus-encoded RNA-dependent RNA replicase distinguishes each family.

Virion Structure

The T = 4 structural features of the tetraviruses (Fig. 19.11) is indicated by cryo-EM reconstructions and X-ray crystallography, including that of NωV and Providence virus (PrV), a *Carmotetravirus*.[222,297] The tetravirus particle contains 240 capsid protein subunits. Twelve chemically identical capsid proteins comprise each triangular face of the virion. The T = 4 capsid arrangement is readily distinguished from the T = 3 symmetry of the smaller nodaviruses that have 60 fewer capsid proteins per particle (Fig. 19.11). Remarkably, the tertiary structures of the

capsid proteins of the virus families are similar. Moreover, the NωV capsid precursor is cleaved after Asn^{570} near the C-terminus in a postassembly mechanism that generates capsid protein β and a smaller peptide γ in a pattern analogous to the required maturation cleavage of the nodavirus capsid precursors (see above). The surface exposure of the lipophilic nodavirus γ peptide disrupts membranes,[10,232] suggesting a comparable role for tetravirus virus membrane penetration. The capsid precursor of PrV is also autocatalytically cleaved to produce a larger β capsid protein and a small C-terminal γ peptide. However, the PrV γ peptide has a structure and function unlike that of NωV.[297] Instead, the PrV γ peptide contributes to genomic RNA binding within the virion particle, like the nodavirus γ peptide. The striking similarities between the PrV and nodavirus γ peptides suggest that PrV is more closely related to the T=3 nodaviruses. It has been suggested that the tetraviruses and nodaviruses may have evolved from a common ancestor.[158,297]

Tetravirus Genome Organization

The tetravirus RNA genome is either mono- or bipartite. The single, monopartite RNAs range in size from 5.6 to 6.6 kb, contain a 7-methyl GpppN cap structure at the 5′ end, and possess a unique tRNA-like structure at the 3′ end.[73] The monopartite genome of NβV has two large ORFs. The 5′ proximal ORF encodes a 214-kDa RNA-dependent replicase–like protein with motifs characteristic of a methyltransferase, nucleotide-binding helicase, and an RNA polymerase. As expected, the RNA replicase–containing polyproteins of the tetraviruses are proteolytically processed.[74] The 3′ proximal NβV ORF encodes the 66-kDa capsid protein. The capsid ORF overlaps the replicase ORF but uses a different (+1) reading frame. It is, therefore, likely that the capsid is translated from a 2.5-kb subgenomic RNA.[114] Indeed, *Thosea asigna* virus (TaV) synthesizes a 2.5-kb subgenomic RNA that encodes the capsid protein.[263]

The omegatetraviruses have a bipartite RNA genome.[73] For example, the bipartite genome of *Helicoverpa armigera* stunt virus (HaSV) consists of RNA1 (5300 bases), which encodes the RNA replicase and smaller proteins, and RNA2 (2478 bases), which encodes the 71-kDa HaSV capsid protein and a smaller protein (p17), which may be involved in packaging of viral RNA during capsid assembly.[73,113] Characteristic of the tetraviruses, the HaSV capsid precursor is cleaved near its C-terminus to generate a ~62-kDa β protein and a smaller 7-kDa γ peptide during assembly. It is unclear whether the omegatetraviruses package both genomic RNAs into a single virion, like the nodaviruses.

Tetravirus Replication, Transmission, and Pathology

Tetraviruses multiply in the cytoplasm of infected cells whereupon crystalline arrays of virus particles accumulate within vesicles.[140] Before permissive cell lines were identified, replication studies were limited to ectopic expression of individual virus genes in heterologous cell lines.[292] Recently, *Helicoverpa armigera* stunt virus (HaSV) was found to multiply productively in cultured *Spodoptera frugiperda* Sf9 cells.[254] Limited multiplication of PrV also occurs in lepidopteran cell lines and newly synthesized genomic and subgenomic RNAs, as well as virus-encoded capsid proteins, are detected.[297] As expected, tetravirus replication occurs on host membranes in the cytoplasm.[73] The availability of permissive cell lines will allow further study of these RNA viruses.

Currently, the tetraviruses are the only known RNA viruses restricted to insects, specifically those within the order Lepidoptera (PrV may represent an exception). Larval infection can be initiated by the ingestion of virus-contaminated foods.[134] Virus multiplication is often restricted to the foregut and midgut cells, even upon injection of infectious virus into the hemolymph of larvae. Virus-mediated killing of midgut cells may account for the stunting pathology associated with HaSV. Susceptible insect larvae exhibit a wide range of pathogenic effects that include rapid death, an extended wasting disease, or a delay in pupation.[74] Death occurs within 4-7 days and is usually accompanied by discoloration, flaccidity, and sometimes liquefaction. Tetravirus infections occur sporadically in natural populations. Thus, ecological aspects of virus spread and the existence of virus reservoirs are poorly understood. Nevertheless, these insect viruses are natural biopesticides that have been observed to control certain insects and several viruses, including *Darna trima* virus and *Thosea asigna* virus, have been successfully applied as biopesticides in oil palm plantation settings.[112]

Dicistroviridae

Classification and Structure

Members of *Dicistroviridae* are small, nonenveloped RNA viruses named for the unusual bicistronic arrangement of their single genomic RNA (Table 19.1). Although these icosahedral insect viruses resemble the vertebrate picornaviruses, they form a taxonomically distinct family.[28,55,143] The best-characterized dicistrovirus is cricket paralysis virus (CrPV), the type species within the *Cripavirus* genus of *Dicistroviridae*. Other cripaviruses include the well-studied dipteran *Drosophila* C virus, hemipteran (aphids and leafhoppers) *Rhopalosiphum padi* virus, and hymenopteran (bees and wasps) Black Queen Cell virus. The dicistroviruses, including Israeli acute paralysis virus, have received increased attention recently due to their association with colony collapse disorder in honeybees.[28,117]

The physicochemical properties of the *Cripavirus* virion are strikingly similar to those of the vertebrate picornaviruses. The icosahedral virion (<40 nm diameter, ρ = 1.35-1.37 g/mL) contains four capsid proteins (VP1, VP2, VP3, and VP4) that are arranged in a pseudo T = 3 lattice with 60 protomers per particle. High-resolution crystal structures of several dicistroviruses, including that of CrPV,[315] show a picornavirus-like capsid conformation and morphology.[348] The proteolytic processing of VP0 → VP3 + VP4 appears to be autocatalytic and VP4 is found in the infectious particle. However, unlike the picornaviruses, CrPV lacks the deep depression (canyon) at the fivefold axis or a VP1 pocket.[315] Also, the residues of VP4 are located between those of VP2 and VP3. Thus, *Cripavirus* assembly may more closely resemble that of the insect nodaviruses and tetraviruses rather than the vertebrate picornaviruses.[315]

Genome Organization and Novel Translation Initiation

Dicistroviruses possess a single-stranded, messenger-sense RNA genome of 8-10 kb. The genome is distinguished by its bicistronic organization of two large ORFs (Fig. 19.13). Upon translation, each ORF produces two different polyproteins, which are proteolytically processed.[28,143,348] Preceded by a 5′ UTR, ORF1 encodes proteins with predicted RNA helicase, picornavirus 3C-like protease, and RNA-dependent RNA polymerase activities. The ORF1 polyprotein is processed by the activity of the 3C-like protease (Fig. 19.13). In addition, the N-terminal end of ORF1 of CrPV and DCV encodes novel RNAi suppressors.[225] These suppressors act by distinct mechanisms but represent a common function

encoded by insect viruses, including the nodaviruses (see above). The capsid proteins are encoded by ORF2, which is preceded by a ~200-nucleotide intergenic UTR that possesses a novel IRES (see below). Thus, unlike the picornaviruses, the capsid proteins and nonstructural replication proteins are encoded by the 3′ and 5′ halves of the genome, respectively (Fig. 19.13). To date, dicistrovirus replication mechanisms are derived mostly from studies of DCV infections of *Drosophila* cell lines. Also, full-length infectious clones are now available for several viruses. As expected, viral RNA replication is mediated by the virus-encoded RNA-dependent RNA polymerase on the membranes of infected cells. Mechanistic details remain to be uncovered.

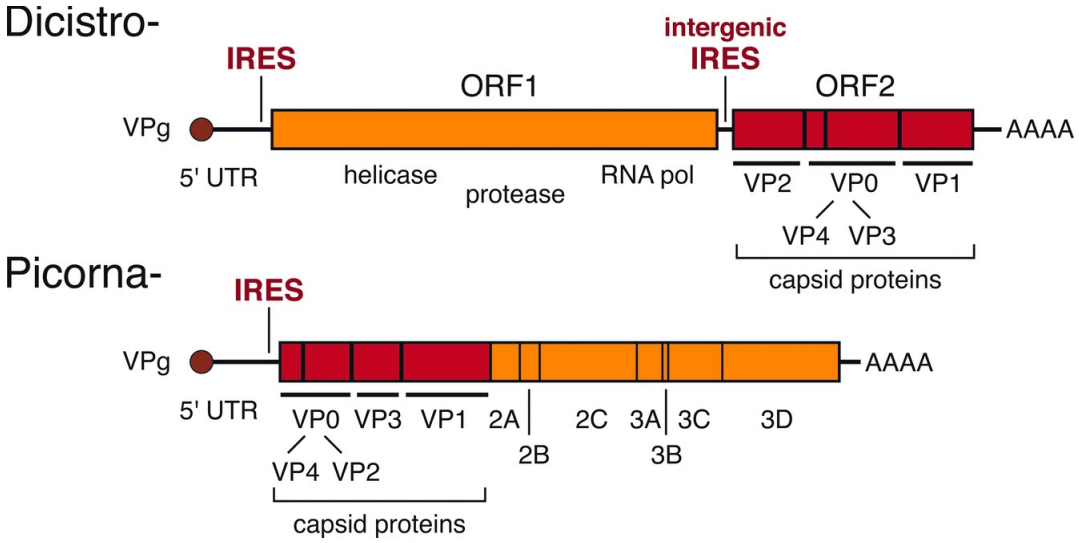

FIGURE 19.13 **Genome organization of the dicistroviruses**. The single-stranded RNA genome of the dicistroviruses, including CrPV and DCV, possess two open reading frames (ORF1 and ORF2) separated by a ~200 nucleotide spacer. An internal ribosome entry site (IRES) precedes both ORFs. ORF1 encodes virus replicative functions (helicase, protease, and RNA-dependent RNA polymerase), whereas ORF2 encodes capsid proteins VP0, VP1, and VP2. VP3 and VP4 are generated by processing of precursor VP0.[315] The small protein VPg and an untranslated region (5′ UTR) are located at the 5′ end of the genomic RNA. By contrast, the vertebrate picornavirus genome possesses a single ORF and must, therefore, produce equimolar levels capsid proteins and replicative functions from the 5′ and 3′ ends of the genome, respectively.

During infection, the dicistrovirus capsid proteins are produced in great excess compared to the nonstructural proteins despite being encoded by the 3′ half of the messenger-active genome. Accounting for this difference, an internal ribosome entry site (IRES) regulates translational initiation from each of the two ORFs. Characterized from CrPV, DCV, *Rhopalosiphum padi* virus, *Plautia stali* intestine virus, and other viruses, these structures are common features of the *Dicistroviridae* and have provided novel insight into mechanisms of translational initiation.[143,348] The use of an IRES avoids the need for a 5′ 7-methyl GpppN cap on viral mRNAs, thereby allowing the virus to escape host- or virus-mediated shutdown of cap-dependent protein synthesis during infection. Thus, invertebrates and vertebrates share these antivirus mechanisms. The intergenic IRES preceding ORF2 (Fig. 19.13) is particularly interesting in that unlike a typical picornavirus IRES, this dicistrovirus IRES does not require any host initiation factors and it initiates translation at a non-AUG codon.[143,348] Rather, the intergenic IRES assembles an 80S ribosome in the absence of initiator Met-tRNA$_i$ or canonical initiation factors to initiate protein synthesis from the second triplet (GCU), which is positioned within the A site of the ribosome. This novel mechanism for IRES-mediated translation initiation involves a pseudoknot structure within the IRES that substitutes for required interactions between Met-tRNA$_i$ and the P-site initiator codon.[143,348] Thus, the intergenic dicistrovirus IRES has provided important understanding of the molecular mechanisms of IRES function.

Dicistrovirus Prevalence and Transmission

The dicistroviruses are widely disseminated in animal populations.[28,143] CrPV was originally isolated from Australian crickets *Teleogryllus oceanicus* and *Teleogryllus commodus*; the CrPV name is derived by its capacity to cause paralysis of the hind limbs of early-instar cricket nymphs. Interestingly, CrPV has one of the widest experimental host ranges of any of the invertebrate viruses and can infect diverse species of multiple insect orders, including those of Diptera, Lepidoptera, Orthoptera, Hymenoptera, and Hemiptera.[56] Experimental inoculation by injection leads to virus multiplication and death of multiple insect species. CrPV transmission can also occur *per os* through contaminated food sources. In contrast to CrPV, DCV has a more restricted host range. DCV has been isolated from wild or laboratory strains of *Drosophila melanogaster*.[56] It can be transmitted *per os* to adult flies or larvae and cause death. In addition, DCV is transmitted horizontally between infected and uninfected adults. Thus, the transmission routes and pathology of dicistroviruses among insects is highly variable but most often follows a fecal-oral route.

Perspectives

Considering the abundance and diversity of insect life on earth, it is likely that only a small fraction of the potential viruses that infect insects have been discovered thus far. Metagenomic analyses promise the discovery of many new viruses of insects. Nonetheless, studies of the viral pathogens characterized to date have revealed novel mechanisms for virus replication, have identified unique viral genes with functions important in human disease (cancer, neurodegeneration, immunodeficiency), and have provided highly useful applications for biotechnology, insect pest management, and gene therapy. It is fully expected that future studies of the insect viruses will uncover fundamental aspects of host-virus interactions, including novel strategies by which these prolific viruses modify and exploit host processes to enhance multiplication and evade antiviral responses. On the basis that many of these responses are conserved in mammals, such studies will provide important insight into the mechanisms of antiviral defenses and virus pathogenicity in humans. In light of the expansion of the natural range of many arthropod vectors due to climate change and the encroachment of humans into new ecological domains, insect viruses will play an even more important

role in human disease and agriculture. Because of this likelihood, along with the enormous contributions of insect viruses to basic biology and biotechnology, there is a critical need for funding for continuing the studies of the insect viruses and their hosts at the molecular level.

REFERENCES

1. Aliyari R, Wu Q, Li HW, et al. Mechanism of induction and suppression of antiviral immunity directed by virus-derived small RNAs in *Drosophila*. Cell Host Microbe. 2008;4(4):387-397.

2. Asgari S. A caspase-like gene from *Heliothis virescens* ascovirus (HvAV-3e) is not involved in apoptosis but is essential for virus replication. Virus Res. 2007;128(1-2):99-105.

3. Asgari S, Johnson KN, eds. Insect Virology. Caister Academic Press; 2010:169-195.

4. Asgari S, Sullivan CS. Role of microRNAs as regulators of host-virus interactions. In: Asgari S, Johnson KN, eds. Insect Virology. Caister Academic Press; 2010:347-366.

5. Ayres MD, Howard SC, Kuzio J, Lopez-Ferber M, Possee RD. The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. Virology. 1994;202(2):586-605.

6. Ball LA. Cellular expression of a functional nodavirus RNA replicon from vaccinia virus vectors. J Virol. 1992;66:2335-2345.

7. Ball LA. Replication of the genomic RNA of a positive-strand RNA animal virus from negative-sense transcripts. Proc Natl Acad Sci U S A. 1994;91(26):12443-12447.

8. Ball LA. Requirements for the self-directed replication of flock house virus RNA 1. J Virol. 1995;69(2):720-727 [published erratum appears in J Virol. 69:2722].

9. Ball LA, Johnson KL. Nodaviruses of insects. In: Miller LK, Ball LA, eds. The Insect Viruses. Plenum Publishing Corporation; 1998:225-267.

10. Banerjee M, Khayat R, Walukiewicz HE, Odegard AL, Schneemann A, Johnson JE. Dissecting the functional domains of a nonenveloped virus membrane penetration peptide. J Virol. 2009;83(13):6929-6933.

11. Banerjee M, Speir JA, Johnson JE. Structural comparison of insect RNA viruses. In: Asgari S, Johnson KN, eds. Insect Virology. Caister Academic Press; 2010:327-346.

12. Bauser CA, Elick TA, Fraser MJ. Characterization of hitchhiker, a transposon insertion frequently associated with baculovirus FP mutants derived upon passage in the TN-368 cell line. Virology. 1996;216:235-237.

13. Beames B, Summers MD. Location and nucleotide sequence of the 25K protein missing from baculovirus few polyhedra (FP) mutants. Virology. 1989;168(2):344-353.

14. Beames B, Summers MD. Sequence comparison of cellular and viral copies of host cell DNA insertions found in *Autographa californica* nuclear polyhedrosis virus. Virology. 1990;174(2):354-363.

15. Beniya H, Funk CJ, Rohrmann GF, Weaver RF. Purification of a virus-induced RNA polymerase from *Autographa californica* nuclear polyhedrosis virus-infected *Spodoptera frugiperda* cells that accurately initiates late and very late transcription in vitro. Virology. 1996;216(1):12-19.

16. Bertin J, Mendrysa SM, LaCount DJ, et al. Apoptotic suppression by baculovirus P35 involves cleavage by and inhibition of a virus-induced CED-3/ICE-like protease. J Virol. 1996;70(9):6251-6259.

17. Best SM. Viral subversion of apoptotic enzymes: escape from death row. Annu Rev Microbiol. 2008;62:171-192.

18. Bezier A, Annaheim M, Herbiniere J, et al. Polydnaviruses of braconid wasps derive from an ancestral nudivirus. Science. 2009;323(5916):926-930.

19. Bezier A, Herbiniere J, Lanzrein B, Drezen JM. Polydnavirus hidden face: the genes producing virus particles of parasitic wasps. J Invertebr Pathol. 2009;101(3):194-203.

20. Bideshi DK, Tan Y, Bigot Y, Federici BA. A viral caspase contributes to modified apoptosis for virus transmission. Genes Dev. 2005;19(12):1416-1421.

21. Bigot Y, Renault S, Nicolas J, et al. Symbiotic virus at the evolutionary intersection of three types of large DNA viruses; iridoviruses, ascoviruses, and ichnoviruses. PLoS One. 2009;4(7):e6397.

22. Birnbaum MJ, Clem RJ, Miller LK. An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. J Virol. 1994;68(4):2521-2528.

23. Black BC, Brennan LA, Dierks PM, Gard IE. Commercialization of baculovirus insecticides. In: Miller LK, ed. The Baculoviruses. Plenum Publishing Corporation; 1997: 341-381.

24. Blissard GW, Kogan PH, Wei R, Rohrmann GF. A synthetic early promoter from a baculovirus: roles of the TATA box and conserved start site CAGT sequence in basal levels of transcription. Virology. 1992;190:783-793.

25. Blissard GW, Rohrmann GF. Baculovirus gp64 gene expression: analysis of sequences modulating early transcription and transactivation by IE1. J Virol. 1991;65(11):5820-5827.

26. Blissard GW, Theilmann DA. Baculovirus entry and egress from insect cells. Annu Rev Virol. 2018;5(1):113-139.

27. Bonning BC. The insect virome: opportunities and challenges. Curr Issues Mol Biol. 2020;34:1-12.

28. Bonning BC, Miller WA. Dicistroviruses. Annu Rev Entomol. 2010;55:129-150.

29. Bonning BC, Nusawardani T. Introduction to the use of baculoviruses as biological insecticides. Methods Mol Biol. 2007;388:359-366.

30. Bonning BC, Saleh MC. The interplay between viruses and RNAi pathways in insects. Annu Rev Entomol. 2021;66:61-79.

31. Bossert M, Carstens EB. Sequential deletion of AcMNPV homologous regions leads to reductions in budded virus production and late protein expression. Virus Res. 2018;256:125-133.

32. Braunagel SC, Burks JK, Rosas-Acosta G, Harrison RL, Ma H, Summers MD. Mutations within the *Autographa californica* nucleopolyhedrovirus FP25K gene decrease the accumulation of ODV-E66 and alter its intranuclear transport. J Virol. 1999;73(10):8559-8570.

33. Braunagel SC, Cox V, Summers MD. Baculovirus data suggest a common but multifaceted pathway for sorting proteins to the inner nuclear membrane. J Virol. 2009;83(3):1280-1288.

34. Braunagel SC, Parr R, Belyavskyi M, Summers MD. *Autographa californica* nucleopolyhedrovirus infection results in Sf9 cell cycle arrest at G2/M phase. Virology. 1998;244(1): 195-211.

35. Broussard DR, Guarino LA, Jarvis DL. Mapping functional domains in AcMNPV pp31. Virology. 1996;222(2):318-331.

36. Bump NJ, Hackett M, Hugunin M, et al. Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35. Science. 1995;269(5232):1885-1888.

37. Burand JP. Nudiviruses. In: Miller LK, Ball LA, eds. The Insect Viruses. Plenum Publishing Corporation; 1998:69-90.

38. Byers NM, Vandergaast RL, Friesen PD. Baculovirus inhibitor-of-apoptosis Op-IAP3 blocks apoptosis by interaction with and stabilization of a host insect cellular IAP. J Virol. 2016;90(1):533-544.

39. Callus BA, Vaux DL. Caspase inhibitors: viral, cellular and chemical. Cell Death Differ. 2007;14(1):73-78.

40. Carson DD, Guarino LA, Summers MD. Functional mapping of an AcNPV immediately early gene which augments expression of the IE-1 trans-activated 39K gene. Virology. 1988;162(2):444-451.

41. Carstens EB, Wu Y. No single homologous repeat region is essential for DNA replication of the baculovirus *Autographa californica* multiple nucleopolyhedrovirus. J Gen Virol. 2007;88(Pt 1):114-122.

42. Cary LC, Goebel M, Corsaro BG, Wang HG, Rosen E, Fraser MJ. Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. Virology. 1989;172(1):156-169.

43. Cerio RJ, Vandergaast R, Friesen PD. Host insect inhibitor-of-apoptosis SfIAP functionally replaces baculovirus IAP but is differentially regulated by Its N-terminal leader. J Virol. 2010;84(21):11448-11460.

44. Chambers AC, Aksular M, Graves LP, Irons SL, Possee RD, King LA. Overview of the baculovirus expression system. Curr Protoc Protein Sci. 2018;91:5.4.1-5.4.6.

45. Chandler D, Bailey AS, Tatchell GM, Davidson G, Greaves J, Grant WP. The development, regulation and use of biopesticides for integrated pest management. Philos Trans R Soc Lond B Biol Sci. 2011;366(1573):1987-1998.

46. Chao JA, Lee JH, Chapados BR, Debler EW, Schneemann A, Williamson JR. Dual modes of RNA-silencing suppression by Flock House virus protein B2. Nat Struct Mol Biol. 2005;12(11):952-957.

47. Chao YC, Lee ST, Chang MC, et al. A 2.9-kilobase noncoding nuclear RNA functions in the establishment of persistent Hz-1 viral infection. J Virol. 1998;72(3): 2233-2245.

48. Charlton CA, Volkman LE. Sequential rearrangement and nuclear polymerization of actin in baculovirus-infected *Spodoptera frugiperda* cells. J Virol. 1991;65(3):1219-1227.

49. Chen CJ, Quentin ME, Brennan LA, Kukel C, Thiem SM. *Lymantria dispar* nucleopolyhedrovirus *hrf-1* expands the larval host range of *Autographa californica* nucleopolyhedrovirus. J Virol. 1998;72(3):2526-2531.

50. Chen CY, Lin CY, Chen GY, Hu YC. Baculovirus as a gene delivery vector: recent understandings of molecular alterations in transduced cells and latest applications. Biotechnol Adv. 2011;29(6):618-631.

51. Chen YR, Zhong S, Fei Z, et al. The transcriptome of the baculovirus *Autographa californica* multiple nucleopolyhedrovirus in Trichoplusia ni cells. J Virol. 2013;87(11): 6391-6405.

52. Cheng CH, Liu SM, Chow TY, et al. Analysis of the complete genome sequence of the Hz-1 virus suggests that it is related to members of the Baculoviridae. J Virol. 2002;76(18):9024-9034.

53. Cheng RH, Reddy VS, Olson NH, Fisher AJ, Baker TS, Johnson JE. Functional implications of quasi-equivalence in a T = 3 icosahedral animal virus established by cryo-electron microscopy and X-ray crystallography. Structure. 1994;2(4):271-282.

54. Chisholm GE, Henner DJ. Multiple early transcripts and splicing of the *Autographa californica* nuclear polyhedrosis virus IE-1 gene. J Virol. 1988;62(9):3193-3200.

55. Christian P, Carstens E, Domier L, et al. Dicistroviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, eds. Virus Taxonomy, 8th Report of the International Committee on Taxonomy of Viruses. Elsevier; 2005.

56. Christian PD, Scotti PD. Picornalike viruses of insects. In: Miller LK, Ball LA, eds. The Insect Viruses. Plenum Publishing Corporation; 1998:301-336.

57. Clarke TE, Clem RJ. In vivo induction of apoptosis correlating with reduced infectivity during baculovirus infection. J Virol. 2003;77(3):2227-2232.

58. Clem RJ. Baculoviruses and apoptosis: a diversity of genes and responses. Curr Drug Targets. 2007;8(10):1069-1074.

59. Clem RJ. Viral IAPs, then and now. Semin Cell Dev Biol. 2015;39:72-79.

60. Clem RJ, Fechheimer M, Miller LK. Prevention of apoptosis by a baculovirus gene during infection of insect cells. Science. 1991;254(5036):1388-1390.

61. Clem RJ, Miller LK. Apoptosis reduces both the in vitro replication and the in vivo infectivity of a baculovirus. J Virol. 1993;67:3730-3738.

62. Clem RJ, Miller LK. Control of programmed cell death by the baculovirus genes *p35* and *iap*. Mol Cell Biol. 1994;14(8):5212-5222.

63. Clem RJ, Passarelli AL. Baculoviruses: sophisticated pathogens of insects. PLoS Pathog. 2013;9(11):e1003729.

64. Cochran MA, Faulkner P. Location of homologous DNA sequences interspersed at five regions in the baculovirus *Autographa californica* nuclear polyhedrosis virus genome. J Virol. 1983;45(3):961-970.

65. Croizier G, Croizier L, Argaud O, Poudevigne D. Extension of *Autographa californica* nuclear polyhedrosis virus host range by interspecific replacement of a short DNA sequence in the p143 helicase gene. Proc Natl Acad Sci U S A. 1994;91(1):48-52.

66. Crouch EA, Passarelli AL. Genetic requirements for homologous recombination in *Autographa californica* nucleopolyhedrovirus. J Virol. 2002;76(18):9323-9334.

67. Cui L, Webb BA. Homologous sequences in the *Campoletis sonorensis* polydnavirus genome are implicated in replication and nesting of the W segment family. J Virol. 1997;71(11):8504-8513.

68. Dalrymple NA. Conserved Baculovirus transcriptional regulators function through distinct viral enhancer-binding mechanisms to stimulate viral promoters. *PhD Thesis, Department of Biochemistry, University of Wisconsin-Madison*. 2009.

69. dela Cruz WP, Friesen PD, Fisher AJ. Crystal structure of baculovirus P35 reveals a novel conformational change in the reactive site loop after caspase cleavage. J Biol Chem. 2001;276(35):32933-32939.

70. Denli AM, Hannon GJ. RNAi: an ever-growing puzzle. Trends Biochem Sci. 2003;28(4):196-201.

71. Dickson JA, Friesen PD. Identification of upstream promoter elements mediating early transcription from the 35,000-molecular-weight protein gene of *Autographa californica* nuclear polyhedrosis virus. J Virol. 1991;65(8):4006-4016.

72. Ding SW, Voinnet O. Antiviral immunity directed by small RNAs. Cell. 2007;130(3): 413-426.

73. Dorrington RA, Jiwaji M, Awando JA, Bruyn MM. Advances in tetravirus research: new insight into the infectious virus lifecycle and an expanding host range. Curr Issues Mol Biol. 2020;34:145-162.

74. Dorrington RA, Short JR. Tetraviruses. In: Asgari S, Johnson KN, eds. Insect Virology. Caister Academic Press; 2010:283-306.

75. Drezen JM, Bezier A, Burke GR, Strand MR. Bracoviruses, ichnoviruses, and virus-like particles from parasitoid wasps retain many features of their virus ancestors. Curr Opin Insect Sci. 2022;49:93-100.

76. Drezen JM, Josse T, Bezier A, Gauthier J, Huguet E, Herniou EA. Impact of lateral transfers on the genomes of Lepidoptera. *Genes* (*Basel*). 2017;8(11):315.

77. Du Q, Lehavi D, Faktor O, Qi Y, Chejanovsky N. Isolation of an apoptosis suppressor gene of the *Spodoptera littoralis* nucleopolyhedrovirus. J Virol. 1999;73(2):1278-1285.

78. Edson KM, Vinson SB, Stoltz DB, Summers MD. Virus in a parasitoid wasp: suppression of the cellular immune response in the parasitoid's host. Science. 1981;211(4482): 582-583.

79. Espagne E, Dupuy C, Huguet E, et al. Genome sequence of a polydnavirus: insights into symbiotic virus evolution. Science. 2004;306(5694):286-289.

80. Fath-Goodin A, Kroemer JA, Webb BA. The *Campoletis sonorensis* ichnovirus vankyrin protein P-vank-1 inhibits apoptosis in insect Sf9 cells. Insect Mol Biol. 2009;18(4): 497-506.

81. Federici BA. A new type of insect pathogen in larvae of the Clover cutworm *Scotogramma trifolii*. J Invertebr Pathol. 1982;40:41-54.

82. Federici BA. Enveloped double-stranded DNA insect virus with novel structure and cytopathology. Proc Natl Acad Sci U S A. 1983;80:7664-7668.

83. Federici BA. Viral pathobiology in relation to insect control. In: Beckage NE, Thompson SN, Federici BA, eds. Parasites and Pathogens of Insects. Vol 2: Pathogens. Academic Press, Inc.; 1993:81-101.

84. Federici BA, Bideshi DK, Tan Y, Bigot Y. Ascoviruses: superb manipulators of apoptosis for viral replication and transmission. In: Van Etten JL, ed. Current Topics in Microbiology and Immunology. Vol 328. Springer-Verlag; 2009:171-195.

85. Federici BA, Hamm JJ, Styer EL. Ascoviridae. In: Adams JR, Bonami JR, eds. Atlas of Invertebrate Viruses. CRC Press; 1991:339-349.

86. Fisher AJ, Cruz W, Zoog SJ, Schneider CL, Friesen PD. Crystal structure of baculovirus P35: role of a novel reactive site loop in apoptotic caspase inhibition. EMBO J. 1999;18(8):2031-2039.

87. Fisher AJ, Johnson JE. Ordered duplex RNA controls capsid architecture in an icosahedral animal virus. Nature. 1993;361:176-179.

88. Fleming JG, Summers MD. Polydnavirus DNA is integrated in the DNA of its parasitoid wasp host. Proc Natl Acad Sci U S A. 1991;88(21):9770-9774.

89. Fraser MJ. Ultrastructural observations of virion maturation in *Autographa californica* nuclear polyhedrosis virus infected *Spodoptera frugiperda* cell cultures. J Ultrastruct Mol Struct Res. 1986;95(1-3):189-195.

90. Fraser MJ, Cary L, Boonvishudhi K, Wang HH. Assay for movement of lepidopteran transposon IFP2 in insect cells using a baculovirus genome as a target DNA. Virology. 1995;211:397-407.

91. Fraser MJ, Smith GE, Summers MD. Acquisition of host cell DNA sequences by baculoviruses: relationship between host DNA insertions and FP mutants of *Autographa californica* nuclear polyhedrosis virus and *Galleria mellonella* nuclear polyhedrosis viruses. J Virol. 1983;47(2):287-300.

92. Friesen PD. Invertebrate transposable elements in the baculovirus genome: characterization and significance. In: Beckage NE, Thompson SN, Federici BA, eds. Parasites and Pathogens of Insects. Academic Press; 1993:147-178.

93. Friesen PD. Regulation of baculovirus early gene expression. In: Miller LK, ed. The Baculoviruses. Plenum Publishing Corporation; 1997:141-166.

94. Friesen PD, Miller LK. Temporal regulation of baculovirus RNA: overlapping early and late transcripts. J Virol. 1985;54(2):392-400.

95. Friesen PD, Nissen MS. Gene organization and transcription of TED, a lepidopteran retrotransposon integrated within the baculovirus genome. Mol Cell Biol. 1990;10: 3067-3077.

96. Friesen PD, Rueckert RR. Synthesis of black beetle virus proteins in cultured *Drosophila* cells: differential expression of RNAs 1 and 2. J Virol. 1981;37:876-886.

97. Friesen PD, Rueckert RR. Black beetle virus: messenger for protein B is a subgenomic viral RNA. J Virol. 1982;42:986-995.

98. Friesen PD, Rueckert RR. Early and late functions in a bipartite RNA virus: evidence for translational control by competition between viral mRNAs. J Virol. 1984;49: 116-124.

99. Fuchs Y, Steller H. Programmed cell death in animal development and disease. Cell. 2011;147(4):742-758.

100. Furuichi Y, Miura K. A blocked structure at the 5′ terminus of mRNA from cytoplasmic polyhedrosis virus. Nature. 1975;253(5490):374-375.

101. Galiana-Arnoux D, Dostert C, Schneemann A, Hoffmann JA, Imler JL. Essential function in vivo for Dicer-2 in host defense against RNA viruses in *Drosophila*. Nat Immunol. 2006;7(6):590-597.

102. Gallagher TM, Friesen PD, Rueckert RR. Autonomous replication and expression of RNA 1 from black beetle virus. J Virol. 1983;46:481-489.

103. Gallagher TM, Rueckert RR. Assembly-dependent maturation cleavage in provirions of a small icosahedral insect ribovirus. J Virol. 1988;62(9):3399-3406.

104. Ghosh S, Jain A, Mukherjee B, Habib S, Hasnain SE. The host factor polyhedrin promoter binding protein (PPBP) is involved in transcription from the baculovirus polyhedrin gene promoter. J Virol. 1998;72(9):7484-7493.

105. Gilbert C, Chateigner A, Ernenwein L, et al. Population genomics supports baculoviruses as vectors of horizontal transfer of insect transposons. Nat Commun. 2014;5:3348.

106. Gilbert C, Peccoud J, Chateigner A, Moumen B, Cordaux R, Herniou EA. Continuous influx of genetic material from host to virus populations. PLoS Genet. 2016;12(2):e1005838.

107. Gilbert C, Peccoud J, Cordaux R. Transposable elements and the evolution of insects. Annu Rev Entomol. 2021;66:355-372.

108. Glocker B, Hoopes RR Jr, Hodges L, Rohrmann GF. In vitro transcription from baculovirus late gene promoters: accurate mRNA initiation by nuclear extracts prepared from infected *Spodoptera frugiperda* cells. J Virol. 1993;67:3771-3776.

109. Goic B, Vodovar N, Mondotte JA, et al. RNA-mediated interference and reverse transcription control the persistence of RNA viruses in the insect model Drosophila. Nat Immunol. 2013;14(4):396-403.

110. Goley ED, Ohkawa T, Mancuso J, et al. Dynamic nuclear actin assembly by Arp2/3 complex and a baculovirus WASP-like protein. Science. 2006;314(5798):464-467.

111. Gordon JD, Carstens EB. Phenotypic characterization and physical mapping of a temperature-sensitive mutant of *Autographa californica* nuclear polyhedrosis virus defective in DNA synthesis. Virology. 1984;138:69-81.

112. Gordon KHJ, Hanzlik TN. Tetraviruses. In: Miller LK, Ball LA, eds. The Insect Viruses. Plenum Publishing Corporation; 1998:269-295.

113. Gordon KHJ, Johnson KN, Hanzlik TN. The larger genomic RNA of *Helicoverpa armigera* stunt tetravirus encodes the viral RNA polymerase and has a novel 3′-terminal tRNA-like structure. Virology. 1995;208:84-98.

114. Gordon KHJ, Williams MR, Hendry DA, Hanzlik TN. Sequence of the genomic RNA of Nudaurelia beta virus (Tetraviridae) defines a novel virus genome organization. Virology. 1999;258(1):42-53.

115. Graves LP, Hughes LC, Irons SL, Possee RD, King LA. In cultured cells the baculovirus P10 protein forms two independent intracellular structures that play separate roles in occlusion body maturation and their release by nuclear disintegration. PLoS Pathog. 2019;15(6):e1007827.

116. Griffiths CM, Barnett AL, Ayres MD, Windass J, King LA, Possee RD. In vitro host range of *Autographa californica* nucleopolyhedrovirus recombinants lacking functional *p35*, *iap1* or *iap2*. J Gen Virol. 1999;80(Pt 4):1055-1066.

117. Grozinger CM, Flenniken ML. Bee viruses: ecology, pathogenicity, and impacts. Annu Rev Entomol. 2019;64:205-226.

118. Gruber A, Stettler P, Heiniger P, Schumperli D, Lanzrein B. Polydnavirus DNA of the braconid wasp *Chelonus inanitus* is integrated in the wasp's genome and excised only in later pupal and adult stages of the female. J Gen Virol. 1996;77(Pt 11):2873-2879.

119. Grula MA, Buller PL, Weaver RF. a-Amanitin-resistant viral RNA synthesis in nuclei isolated from nuclear polyhedrosis virus-infected *Heliothis zea* larvae and *Spodoptera frugiperda* cells. J Virol. 1981;38(3):916-921.

120. Guarino LA, Ghosh A, Dasmahapatra B, Dasgupta R, Kaesberg P. Sequence of the black beetle virus subgenomic RNA and its location in the viral genome. Virology. 1984;139(1):199-203.

121. Guarino LA, Gonzalez MA, Summers MD. Complete sequence and enhancer function of the homologous DNA regions of *Autographa californica* nuclear polyhedrosis virus. J Virol. 1986;60(1):224-229.

122. Guarino LA, Mistretta TA, Dong W. DNA binding activity of the baculovirus late expression factor PP31. Virus Res. 2002;90(1-2):187-195.

123. Guarino LA, Smith M. Regulation of delayed-early gene transcription by dual TATA boxes. J Virol. 1992;66:3733-3739.

124. Guarino LA, Summers MD. Functional mapping of a trans-activating gene required for expression of a baculovirus delayed-early gene. J Virol. 1986;57(2):563-571.

125. Guarino LA, Summers MD. Interspersed homologous DNA of *Autographa californica* nuclear polyhedrosis virus enhances delayed-early gene expression. J Virol. 1986;60(1): 215-223.

126. Guarino LA, Summers MD. Nucleotide sequence and temporal expression of a baculovirus regulatory gene. J Virol. 1987;61(7):2091-2099.

127. Guarino LA, Xu B, Jin J, Dong W. A virus-encoded RNA polymerase purified from baculovirus-infected cells. J Virol. 1998;72(10):7985-7991.

128. Guibinga GH, Friedmann T. Baculovirus GP64-pseudotyped HIV-based lentivirus vectors are stabilized against complement inactivation by codisplay of decay accelerating factor (DAF) or of a GP64-DAF fusion protein. Mol Ther. 2005;11(4):645-651.

129. Guo YX, Wei T, Dallmann K, Kwang J. Induction of caspase-dependent apoptosis by betanodaviruses GGNNV and demonstration of protein alpha as an apoptosis inducer. Virology. 2003;308(1):74-82.

130. Guy MP, Friesen PD. Reactive-site cleavage residues confer target specificity to baculovirus P49, a dimeric member of the P35 family of caspase inhibitors. J Virol. 2008;82(15): 7504-7514.

131. Gyrd-Hansen M, Meier P. IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. Nat Rev Cancer. 2010;10(8):561-574.

132. Hajek KL, Friesen PD. Proteolytic processing and assembly of gag and gag-pol proteins of TED, a baculovirus-associated retrotransposon of the gypsy family. J Virol. 1998;72(11):8718-8724.

133. Handler AM, O'Brochta DA. Transposable elements for insect transformation. In: Gilberet LI, Iatrou K, Gill SS, eds. Comprehensive Molecular Insect Science. Vol 4. Elsevier; 2005:437-474.

134. Hanzlik TN, Dorrian SJ, Gordon KHJ, Christian PD. A novel small RNA virus isolated from the cotton bollworm, *Helicoverpa armigera*. J Gen Virol. 1993;74:1805-1810.

135. Harrison RL. Structural divergence among genomes of closely related baculoviruses and its implications for baculovirus evolution. J Invertebr Pathol. 2009;101(3):181-186.

136. Harrison RL, Herniou EA, Bezier A, et al. ICTV virus taxonomy profile: Nudiviridae. J Gen Virol. 2020;101(1):3-4.

137. Harrison RL, Jarvis DL. Transforming lepidopteran insect cells for continuous recombinant protein expression. Methods Mol Biol. 2016;1350:329-348.

138. Harrison RL, Jarvis DL, Summers MD. The role of the AcMNPV 25K gene, "FP25", in baculovirus *polh* and *p10* expression. Virology. 1996;226:34-46.

139. Hawtin RE, Zarkowska T, Arnold K, et al. Liquefaction of *Autographa californica* nucleopolyhedrovirus-infected insects is dependent on the integrity of virus-encoded chitinase and cathepsin genes. Virology. 1997;238(2):243-253.

140. Hendry D, Agrawal D. Small RNA viruses of insects: Tetraviridae. In: Webster RG, Granoff A, eds. Encyclopedia of Virology. Academic Press; 1994.

141. Hershberger PA, Dickson JA, Friesen PD. Site-specific mutagenesis of the 35-kilodalton protein gene encoded by *Autographa californica* nuclear polyhedrosis virus: cell line-specific effects on virus replication. J Virol. 1992;66:5525-5533.

142. Hershberger PA, LaCount DL, Friesen PD. The apoptotic suppressor P35 is required early during baculovirus replication and is targeted to the cytosol of infected cells. J Virol. 1994;68:3467-3477.

143. Hertz MI, Thompson SR. Mechanism of translation initiation by Dicistroviridae IGR IRESs. Virology. 2011;411(2):355-361.

144. Hodgson JJ, Arif BM, Krell PJ. Interaction of *Autographa californica* multiple nucleopolyhedrovirus cathepsin protease progenitor (proV-CATH) with insect baculovirus chitinase as a mechanism for proV-CATH cellular retention. J Virol. 2011;85(8):3918-3929.

145. Hodgson JJ, Krell PJ, Passarelli AL. Mature viral cathepsin is required for release of viral occlusion bodies from Autographa californica multiple nucleopolyhedrovirus-infected cells. Virology. 2021;556:23-32.

146. Hoopes RR, Rohrmann GF. In vitro transcription of baculovirus immediate early genes: accurate messenger RNA initiation by nuclear extracts from both insect and human cells. Proc Natl Acad Sci U S A. 1991;88(10):4513-4517.

147. Hoover K, Grove M, Gardner M, Hughes DP, McNeil J, Slavicek J. A gene for an extended phenotype. Science. 2011;333:1401.

148. Hosur MV, Schmidt T, Tucker RC, et al. Structure of an insect virus at 3.0 Å resolution. Proteins. 1987;2:167-176.

149. Hozak RR, Manji GA, Friesen PD. The BIR motifs mediate dominant interference and oligomerization of inhibitor of apoptosis Op-IAP. Mol Cell Biol. 2000;20:1877-1885.

150. Huang N, Wu W, Yang K, Passarelli AL, Rohrmann GF, Clem RJ. Baculovirus infection induces a DNA damage response that is required for efficient viral replication. J Virol. 2011;85(23):12547-12556.

151. Huang Q, Deveraux QL, Maeda S, et al. Evolutionary conservation of apoptosis mechanisms: lepidopteran and baculoviral inhibitor of apoptosis proteins are inhibitors of mammalian caspase-9. Proc Natl Acad Sci U S A. 2000;97:1427-1432.

152. Huh NE, Weaver RF. Identifying the RNA polymerases that synthesize specific transcripts of the *Autographa californica* nuclear polyhedrosis virus. J Gen Virol. 1990;71(Pt 1): 195-201.

153. Hussain M, Abraham AM, Asgari S. An Ascovirus-encoded RNase III autoregulates its expression and suppresses RNA interference-mediated gene silencing. J Virol. 2010;84(7):3624-3630.

154. Ikeda M, Kobayashi M. Cell-cycle perturbation in Sf9 cells infected with *Autographa californica* nucleopolyhedrovirus. Virology. 1999;258(1):176-188.

155. Ince IA, Ozcan O, Ilter-Akulke AZ, Scully ED, Ozgen A. Invertebrate iridoviruses: a glance over the last decade. Viruses. 2018;10(4):161.

156. Jackson TA, Crawford AM, Glare TR. Oryctes virus—time for a new look at a useful biocontrol agent. J Invertebr Pathol. 2005;89(1):91-94.

157. Jarvis DL. Baculovirus-insect cell expression systems. Methods Enzymol. 2009;463: 191-222.

158. Johnson JE, Reddy V. Structural studies of nodaviruses and tetraviruses. In: Miller LK, Ball LA, eds. The Insect Viruses. Plenum Publishing Corporation; 1998:171-221.

159. Johnson KL, Price BD, Eckerle LD, Ball LA. Nodamura virus nonstructural protein B2 can enhance viral RNA accumulation in both mammalian and insect cells. J Virol. 2004;78(12):6698-6704.

160. Johnson RM, Rasgon JL. Densonucleosis viruses ('densoviruses') for mosquito and pathogen control. Curr Opin Insect Sci. 2018;28:90-97.

161. Joshi PRH, Venereo-Sanchez A, Chahal PS, Kamen AA. Advancements in molecular design and bioprocessing of recombinant adeno-associated virus gene delivery vectors using the insect-cell baculovirus expression platform. Biotechnol J. 2021;16(4):e2000021.

162. Jovel J, Schneemann A. Molecular characterization of *Drosophila* cells persistently infected with Flock House virus. Virology. 2011;419(1):43-53.

163. Kadlec J, Loureiro S, Abrescia NG, Stuart DI, Jones IM. The postfusion structure of baculovirus gp64 supports a unified view of viral fusion machines. Nat Struct Mol Biol. 2008;15(10):1024-1030.

164. Kamita SG, Majima K, Maeda S. Identification and characterization of the p35 gene of *Bombyx mori* nuclear polyhedrosis virus that prevents virus-induced apoptosis. J Virol. 1993;67(1):455-463.

165. Kamita SG, Nagasaka K, Chua JW, et al. A baculovirus-encoded protein tyrosine phosphatase gene induces enhanced locomotory activity in a lepidopteran host. Proc Natl Acad Sci U S A. 2005;102(7):2584-2589.

166. Kato T, Kajikawa M, Maenaka K, Park EY. Silkworm expression system as a platform technology in life science. Appl Microbiol Biotechnol. 2010;85(3):459-470.

167. Kogan PH, Blissard GW. A baculovirus gp64 early promoter is activated by host transcription factor binding to CACGTG and GATA elements. J Virol. 1994;68(2):813-822.

168. Kool M, Ahrens CH, Goldbach RW, Rohrmann GF, Vlak JM. Identification of genes involved in DNA replication of the *Autographa*

californica baculovirus. Proc Natl Acad Sci U S A. 1994;91(23):11212-11216.

169. Kool M, Goldbach RW, Vlak JM. A putative non-hr origin of DNA replication in the HindIII-K fragment of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus. J Gen Virol. 1994;75(12):3345-3352.

170. Kool M, Voeten JT, Goldbach RW, Tramper J, Vlak JM. Identification of seven putative origins of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus DNA replication. J Gen Virol. 1993;74(12):2661-2668.

171. Kopek BG, Settles EW, Friesen PD, Ahlquist P. Nodavirus-induced membrane rearrangement in replication complex assembly requires replicase protein a, RNA templates, and polymerase activity. J Virol. 2010;84(24):12492-12503.

172. Korber S, Shaik Syed Ali P, Chen JC. Structure of the RNA-binding domain of Nodamura virus protein B2, a suppressor of RNA interference. *Biochemistry* (*Mosc*). 2009;48(11):2307-2309.

173. Kost TA, Kemp CW. Fundamentals of baculovirus expression and applications. Adv Exp Med Biol. 2016;896:187-197.

174. Kovacs GR, Choi J, Guarino LA, Summers MD. Functional dissection of the *Autographa californica* nuclear polyhedrosis virus immediate-early 1 transcriptional regulatory protein. J Virol. 1992;66:7429-7437.

175. Kovacs GR, Guarino LA, Summers MD. Novel regulatory properties of the IE1 and IE0 transactivators encoded by the baculovirus *Autographa californica* multicapsid nuclear polyhedrosis virus. J Virol. 1991;65(10):5281-5288.

176. Krappa R, Behn-Krappa A, Jahnel F, Doerfler W, Knebel-Mörsdorf D. Differential factor binding at the promoter of early baculovirus gene PE38 during viral infection: GATA motif is recognized by an insect protein. J Virol. 1992;66:3494-3503.

177. Krappa R, Knebel-Mörsdorf D. Identification of the very early transcribed baculovirus gene PE-38. J Virol. 1991;65(2):805-812.

178. Krappa R, Roncarati R, Knebel-Morsdorf D. Expression of PE38 and IE2, viral members of the C3HC4 finger family, during baculovirus infection: PE38 and IE2 localize to distinct nuclear regions. J Virol. 1995;69(9):5287-5293.

179. Kremer A, Knebel-Mörsdorf D. The early baculovirus he65 promoter: on the mechanism of transcriptional activation by IE1. Virology. 1998;249(2):336-351.

180. Kroemer JA, Bonning BC, Harrison RL. Expression, delivery and function of insecticidal proteins expressed by recombinant baculoviruses. Viruses. 2015;7(1):422-455.

181. Kroemer JA, Webb BA. Polydnavirus genes and genomes: emerging gene families and new insights into polydnavirus replication. Annu Rev Entomol. 2004;49:431-456.

182. Kroemer JA, Webb BA. Ikappabeta-related vankyrin genes in the *Campoletis sonorensis* ichnovirus: temporal and tissue-specific patterns of expression in parasitized *Heliothis virescens* lepidopteran hosts. J Virol. 2005;79(12):7617-7628.

183. Lacey LA, Grzywacz D, Shapiro-Ilan DI, Frutos R, Brownbridge M, Goettel MS. Insect pathogens as biological control agents: Back to the future. J Invertebr Pathol. 2015;132: 1-41.

184. LaCount DJ, Friesen PD. Role of early and late replication events in induction of apoptosis by baculoviruses. J Virol. 1997;71(2):1530-1537.

185. LaCount DJ, Hanson SF, Schneider CL, Friesen PD. Caspase inhibitor P35 and inhibitor of apoptosis Op-IAP block *in vivo* proteolytic activation of an effector caspase at different steps. J Biol Chem. 2000;275:15657-15664.

186. Lannan E, Vandergaast R, Friesen PD. Baculovirus caspase inhibitors P49 and P35 block virus-induced apoptosis downstream of effector caspase DrICE activation in *Drosophila melanogaster* cells. J Virol. 2007;81(17):9319-9330.

187. Lee HY, Krell PJ. Generation and analysis of defective genomes of *Autographa californica* nuclear polyhedrosis virus. J Virol. 1992;66:4339-4347.

188. Leisy DJ, Rasmussen C, Kim HT, Rohrmann GF. The *Autographa californica* nuclear polyhedrosis virus homologous region 1a: identical sequences are essential for DNA replication activity and transcriptional enhancer function. Virology. 1995;208(2):742-752.

189. Lepore LS, Roelvink PR, Granados RR. Enhancin, the granulosis virus protein that facilitates nucleopolyhedrovirus (NPV) infections, is a metalloprotease. J Invertebr Pathol. 1996;68(2):131-140.

190. Lerch RA, Friesen PD. The baculovirus-integrated retrotransposon TED encodes gag and pol proteins that assemble into viruslike particles with reverse transcriptase. J Virol. 1992;66(3):1590-1601.

191. Lerch RA, Friesen PD. The 35-kilodalton protein gene (p35) of *Autographa californica* nuclear polyhedrosis virus and the neomycin resistance gene provide dominant selection of recombinant baculoviruses. Nucleic Acids Res. 1993;21(8):1753-1760 [published erratum in *Nucl Acids Res*. 1993;21:2962].

192. Li H, Li WX, Ding SW. Induction and suppression of RNA silencing by an animal virus. Science. 2002;296(5571):1319-1321.

193. Li L, Harwood SH, Rohrmann GF. Identification of additional genes that influence baculovirus late gene expression. Virology. 1999;255(1):9-19.

194. Li WX, Li H, Lu R, et al. Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. Proc Natl Acad Sci U S A. 2004;101(5):1350-1355.

195. Li Z, Blissard GW. Baculovirus GP64 disulfide bonds: the intermolecular disulfide bond of *Autographa californica* multicapsid nucleopolyhedrovirus GP64 is not essential for membrane fusion and virion budding. J Virol. 2010;84(17):8584-8595.

196. Li Z, Fan Y, Wei J, et al. Baculovirus utilizes cholesterol transporter NIEMANN-Pick C1 for host cell entry. Front Microbiol. 2019;10:2825.

197. Lin CL, Lee JC, Chen SS, et al. Persistent Hz-1 virus infection in insect cells: evidence for insertion of viral DNA into host chromosomes and viral infection in a latent status. J Virol. 1999;73(1):128-139.

198. Liu X, Tian Z, Cai L, et al. Baculoviruses hijack the visual perception of their caterpillar hosts to induce climbing behaviour thus promoting virus dispersal. Mol Ecol. 2022;31(9):2752-2765.

199. Loiseau V, Peccoud J, Bouzar C, et al. Monitoring insect transposable elements in large double-stranded DNA viruses reveals host-to-virus and virus-to-virus transposition. Mol Biol Evol. 2021;38(9):3512-3530.

200. Lu A, Carstens EB. Immediate-early baculovirus genes transactivate the p143 gene promoter of *Autographa californica* nuclear polyhedrosis virus. Virology. 1993;195(2):710-718.

201. Lu A, Krell PJ, Vlak JM, Rohrmann GF. Baculovirus DNA replication. In: Miller LK, ed. The Baculoviruses. Plenum Publishing Corporation; 1997:171-191.

202. Lu A, Miller LK. The roles of eighteen baculovirus late expression factor genes in transcription and DNA replication. J Virol. 1995;69:975-982.

203. Lu L, Rivkin H, Chejanovsky N. The immediate-early protein IE0 of the *Autographa californica* nucleopolyhedrovirus is not essential for viral replication. J Virol. 2005;79(15):10077-10082.

204. Lu R, Maduro M, Li F, et al. Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*. Nature. 2005;436(7053):1040-1043.

205. Lübbert H, Doerfler W. Mapping of early and late transcripts encoded by the *Autographa californica* nuclear polyhedrosis virus genome: Is viral RNA spliced? J Virol. 1984;50: 497-506.

206. Malik HS, Henikoff S, Eickbush TH. Poised for contagion: evolutionary origins of the infectious abilities of invertebrate retroviruses. Genome Res. 2000;10(9):1307-1318.

207. Manji GA, Friesen PD. Apoptosis in motion. An apical, P35-insensitive caspase mediates programmed cell death in insect cells. J Biol Chem. 2001;276(20):16704-16710.

208. Manji GA, Hozak RR, LaCount DJ, Friesen PD. Baculovirus inhibitor of apoptosis functions at or upstream of the apoptotic suppressor P35 to prevent programmed cell death. J Virol. 1997;71(6):4509-4516.

209. McLachlin JR, Miller LK. Identification and characterization of *vlf-1*, a baculovirus gene involved in very late gene expression. J Virol. 1994;68(12):7746-7756.

210. Means JC, Muro I, Clem RJ. Silencing of the baculovirus Op-iap3 gene by RNA interference reveals that it is required for prevention of apoptosis during *Orgyia pseudotsugata* M nucleopolyhedrovirus infection of Ld652Y cells. J Virol. 2003;77(8):4481-4488.

211. Means JC, Passarelli AL. Viral fibroblast growth factor, matrix metalloproteases, and caspases are associated with enhancing systemic infection by baculoviruses. Proc Natl Acad Sci U S A. 2010;107(21):9825-9830.

212. Means JC, Penabaz T, Clem RJ. Identification and functional characterization of AMVp33, a novel homolog of the baculovirus caspase inhibitor p35 found in *Amsacta moorei* entomopoxvirus. Virology. 2007;358(2):436-447.

213. Menzel T, Rohrmann GF. Diversity of errantivirus (retrovirus) sequences in two cell lines used for baculovirus expression, *Spodoptera frugiperda* and *Trichoplusia ni*. Virus Genes. 2008;36(3):583-586.

214. Milks ML, Washburn JO, Willis LG, Volkman LE, Theilmann DA. Deletion of pe38 attenuates AcMNPV genome replication, budded virus production, and virulence in *Heliothis virescens*. Virology. 2003;310(2):224-234.

215. Miller DJ, Schwartz MD, Ahlquist P. Flock house virus RNA replicates on outer mitochondrial membranes in Drosophila cells. J Virol. 2001;75(23):11664-11676.

216. Miller DW, Miller LK. A virus mutant with an insertion of a copia-like transposable element. Nature. 1982;299(5883):562-564.

217. Mistretta TA, Guarino LA. Transcriptional activity of baculovirus very late factor 1. J Virol. 2005;79(3):1958-1960.

218. Mitchell JK, Friesen PD. Baculoviruses exploit a pro-apoptotic DNA damage response to promote virus multiplication. *J Virol*. 2012;86(24):13542-13553.

219. Monsma SA, Oomens AG, Blissard GW. The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. J Virol. 1996;70(7):4607-4616.

220. Morris TD, Miller LK. Mutational analysis of a baculovirus major late promoter. Gene. 1994;140(2):147-153.

221. Moscardi F. Assessment of the application of baculoviruses for control of lepidoptera. Annu Rev Entomol. 1999;44:257-289.

222. Munshi S, Liljas L, Cavarelli J, et al. The 2.8 A structure of a T = 4 animal virus and its implications for membrane translocation of RNA. J Mol Biol. 1996;261(1):1-10.

223. Muro I, Hay BA, Clem RJ. The *Drosophila* DIAP1 protein is required to prevent accumulation of a continuously generated, processed form of the apical caspase DRONC. J Biol Chem. 2002;277(51):49644-49650.

224. Nagamine T, Kawasaki Y, Abe A, Matsumoto S. Nuclear marginalization of host cell chromatin associated with expansion of two discrete virus-induced subnuclear compartments during baculovirus infection. J Virol. 2008;82(13):6409-6418.

225. Nayak A, Berry B, Tassetto M, et al. Cricket paralysis virus antagonizes Argonaute 2 to modulate antiviral defense in *Drosophila*. Nat Struct Mol Biol. 2011;17(5):547-554.

226. Nissen MS, Friesen PD. Molecular analysis of the transcriptional regulatory region of an early baculovirus gene. J Virol. 1989;63(2):493-503.

227. O'Brochta DA, Alford RT, Pilitt KL, Aluvihare CU, Harrell RA II. piggyBac transposon remobilization and enhancer detection in *Anopheles* mosquitoes. Proc Natl Acad Sci U S A. 2011;108(39):16339-16344.

228. O'Reilly DR. Auxiliary genes of baculoviruses. In: Miller LK, ed. The Baculoviruses. Plenum Publishing Corporation; 1997:267-300.

229. O'Reilly DR, Miller LK. A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase. Science. 1989;245(4922):1110-1112.

230. O'Reilly DR, Miller LK. Improvement of a baculovirus pesticide by deletion of the egt gene. *Biotechnology* (*N Y*). 1991;9:1086-1089.

231. O'Riordan MX, Bauler LD, Scott FL, Duckett CS. Inhibitor of apoptosis proteins in eukaryotic evolution and development: a model of thematic conservation. Dev Cell. 2008;15(4):497-508.

232. Odegard AL, Kwan MH, Walukiewicz HE, Banerjee M, Schneemann A, Johnson JE. Low endocytic pH and capsid protein autocleavage are critical components of Flock House virus cell entry. J Virol. 2009;83(17):8628-8637.

233. Ohkawa T, Volkman LE. Nuclear F-actin is required for AcMNPV nucleocapsid morphogenesis. Virology. 1999;264(1):1-4.

234. Okano K, Mikhailov VS, Maeda S. Colocalization of baculovirus IE-1 and two DNA-binding proteins, DBP and LEF-3, to viral replication factories. J Virol. 1999;73(1):110-119.

235. Okano K, Vanarsdall AL, Rohrmann GF. Characterization of a baculovirus lacking the alkaline nuclease gene. J Virol. 2004;78(19):10650-10656.

236. Olson VA, Wetter JA, Friesen PD. Oligomerization mediated by a helix-loop-helix-like domain of baculovirus IE1 is required for early promoter transactivation. J Virol. 2001;75(13):6042-6051.

237. Olson VA, Wetter JA, Friesen PD. Baculovirus transregulator IE1 requires a dimeric nuclear localization element for nuclear import and promoter activation. J Virol. 2002;76(18):9505-9515.

238. Olson VA, Wetter JA, Friesen PD. The highly conserved basic domain I of baculovirus IE1 is required for hr enhancer DNA binding and hr-dependent transactivation. J Virol. 2003;77(10):5668-5677.

239. Ono C, Okamoto T, Abe T, Matsuura Y. Baculovirus as a tool for gene delivery and gene therapy. Viruses. 2018;10(9):510.

240. Ooi BG, Miller LK. Regulation of host RNA levels during baculovirus infection. Virology. 1988;166(2):515-523.

241. Ooi BG, Miller LK. Transcription of the baculovirus polyhedrin gene reduces the levels of an antisense transcript initiated downstream. J Virol. 1990;64(6):3126-3129.

242. Oomens AG, Blissard GW. Requirement for GP64 to drive efficient budding of *Autographa californica* multicapsid nucleopolyhedrovirus. Virology. 1999;254(2):297-314.

243. Oomens AG, Wertz GW. The baculovirus GP64 protein mediates highly stable infectivity of a human respiratory syncytial virus lacking its homologous transmembrane glycoproteins. J Virol. 2004;78(1):124-135.

244. Oppenheimer DI, Volkman LE. Evidence for rolling circle replication of *Autographa californica* M nucleopolyhedrovirus genomic DNA. Arch Virol. 1997;142(10):2107-2113.

245. Ozers MS, Friesen PD. The Env-like open reading frame of the baculovirus-integrated retrotransposon TED encodes a retrovirus-like envelope protein. Virology. 1996;226(2): 252-259.

246. Passarelli AL. Barriers to success: how baculoviruses establish efficient systemic infections. Virology. 2011;411(2):383-392.

247. Passarelli AL, Guarino LA. Baculovirus late and very late gene regulation. Curr Drug Targets. 2007;8(10):1103-1115.

248. Passarelli AL, Miller LK. Three baculovirus genes involved in late and very late gene expression: *ie-1*, *ie-n*, and *lef-2*. J Virol. 1993;67(4):2149-2158.

249. Pathakamuri JA, Theilmann DA. The acidic activation domain of the baculovirus transactivator IE1 contains a virus-specific domain essential for DNA replication. J Virol. 2002;76(11):5598-5604.

250. Paudel S, Marshall SDG, Richards NK, Hazelman G, Tanielu P, Jackson TA. Coconut rhinoceros beetle in Samoa: review of a century-old invasion and prospects for control in a changing future. Insects. 2022;13(5):487.

251. Pearson MN, Bjornson RM, Pearson GD, Rohrmann GF. The *Autographa californica* baculovirus genome: evidence for multiple replication

origins. Science. 1992;257:1382-1384.

252. Pearson MN, Rohrmann GF. Transfer, incorporation, and substitution of envelope fusion proteins among members of the Baculoviridae, Orthomyxoviridae, and Metaviridae (insect retrovirus) families. J Virol. 2002;76(11):5301-5304.

253. Pei Z, Reske G, Huang Q, Hammock BD, Qi Y, Chejanovsky N. Characterization of the apoptosis suppressor protein P49 from the *Spodoptera littoralis* nucleopolyhedrovirus. J Biol Chem. 2002;277(50):48677-48684.

254. Penkler DL, Jiwaji M, Domitrovic T, Short JR, Johnson JE, Dorrington RA. Binding and entry of a non-enveloped T=4 insect RNA virus is triggered by alkaline pH. Virology. 2016;498:277-287.

255. Pennock GD, Shoemaker C, Miller LK. Strong and regulated expression of *Escherichia coli* beta-galactosidase in insect cells with a baculovirus vector. Mol Cell Biol. 1984;4(3): 399-406.

256. Perera SC, Wong P, Krell PJ, Arif BM. Expression of heterologous genes in the *Amsacta moorei* entomopoxvirus. J Virol Methods. 2010;165(1):1-8.

257. Petersen JM, Bezier A, Drezen JM, van Oers MM. The naked truth: an updated review on nudiviruses and their relationship to bracoviruses and baculoviruses. J Invertebr Pathol. 2022;189:107718.

258. Poirier EZ, Goic B, Tome-Poderti L, et al. Dicer-2-dependent generation of viral DNA from defective genomes of RNA viruses modulates antiviral immunity in insects. Cell Host Microbe. 2018;23(3):353-365 e8.

259. Possee RD, Chambers AC, Graves LP, Aksular M, King LA. Recent developments in the use of baculovirus expression vectors. Curr Issues Mol Biol. 2020;34:215-230.

260. Price BD, Rueckert RR, Ahlquist P. Complete replication of an animal virus and maintenance of expression vectors derived from it in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A. 1996;93(18):9465-9470.

261. Prikhod'ko EA, Lu A, Wilson JA, Miller LK. In vivo and in vitro analysis of baculovirus ie-2 mutants. J Virol. 1999;73(3):2460-2468.

262. Prikhod'ko EA, Miller LK. Role of baculovirus IE2 and its RING finger in cell cycle arrest. J Virol. 1998;72(1):684-692.

263. Pringle FM, Gordon KH, Hanzlik TN, Kalmakoff J, Scotti PD, Ward VK. A novel capsid expression strategy for *Thosea asigna* virus (Tetraviridae). J Gen Virol. 1999;80(Pt 7): 1855-1863.

264. Pruijssers AJ, Strand MR. PTP-H2 and PTP-H3 from Microplitis demolitor Bracovirus localize to focal adhesions and are antiphagocytic in insect immune cells. J Virol. 2007;81(3):1209-1219.

265. Pullen SS, Friesen PD. The CAGT motif functions as an initiator element during early transcription of the baculovirus transregulator *ie-1*. J Virol. 1995;69(6):3575-3583.

266. Pullen SS, Friesen PD. Early transcription of the *ie-1* transregulator gene of *Autographa californica* nuclear polyhedrosis virus is regulated by DNA sequences within its 5′ noncoding leader region. J Virol. 1995;69(1):156-165.

267. Qi N, Cai D, Qiu Y, et al. RNA binding by a novel helical fold of b2 protein from Wuhan nodavirus mediates the suppression of RNA interference and promotes b2 dimerization. J Virol. 2011;85(18):9543-9554.

268. Quadt I, Mainz D, Mans R, Kremer A, Knebel-Morsdorf D. Baculovirus infection raises the level of TATA-binding protein that colocalizes with viral DNA replication sites. J Virol. 2002;76(21):11123-11127.

269. Rapp JC, Wilson JA, Miller LK. Nineteen baculovirus open reading frames, including LEF-12, support late gene expression. J Virol. 1998;72(12):10197-10206.

270. Rasmussen C, Rohrmann GF. Characterization of the *Spodoptera frugiperda* TATA-binding protein: nucleotide sequence and response to baculovirus infection. Insect Biochem Mol Biol. 1994;24(7):699-708.

271. Ribeiro BM, Hutchinson K, Miller LK. A mutant baculovirus with a temperature-sensitive IE-1 transregulatory protein. J Virol. 1994;68(2):1075-1084.

272. Rodems SM, Friesen PD. The *hr*5 transcriptional enhancer stimulates early expression from the *Autographa californica* nuclear polyhedrosis virus genome but is not required for virus replication. J Virol. 1993;67(10):5776-5785.

273. Rodems SM, Friesen PD. Transcriptional enhancer activity of *hr*5 requires dual-palindrome half sites that mediate binding of a dimeric form of the baculovirus transregulator IE1. J Virol. 1995;69(9):5368-5375.

274. Rodems SM, Pullen SS, Friesen PD. DNA-dependent transregulation by IE1 of *Autographa californica* nuclear polyhedrosis virus: IE1 domains required for transactivation and DNA binding. J Virol. 1997;71(12):9270-9277.

275. Rohrmann GF. Baculovirus Molecular Biology. 4th ed. National Center for Biotechnology Information (US), U.S. National Library of Medicine; 2019. https://www.ncbi.nlm.nih.gov/books/NBK543458/

276. Roncarati R, Knebel-Mörsdorf D. Identification of the early actin-rearrangement-inducing factor gene, *arif-1*, from *Autographa californica* multicapsid nuclear polyhedrosis virus. J Virol. 1997;71(10):7933-7941 [published erratum J Virol. 1998;72:888-889].

277. Sahul Hameed AS, Ninawe AS, Nakai T, Chi SC, Johnson KL; Ictv report C. ICTV virus taxonomy profile: Nodaviridae. J Gen Virol. 2019;100(1):3-4.

278. Salem TZ, Zhang F, Xie Y, Thiem SM. Comprehensive analysis of host gene expression in *Autographa californica* nucleopolyhedrovirus-infected *Spodoptera frugiperda* cells. Virology. 2011;412(1):167-178.

279. Sandoval-Villegas N, Nurieva W, Amberger M, Ivics Z. Contemporary transposon tools: a review and guide through mechanisms and applications of sleeping beauty, piggyBac and Tol2 for genome engineering. Int J Mol Sci. 2021;22(10):5084.

280. Sandro Q, Relizani K, Benchaouir R. AAV Production using baculovirus expression vector system. Methods Mol Biol. 2019;1937:91-99.

281. Schaly S, Ghebretatios M, Prakash S. Baculoviruses in gene therapy and personalized medicine. Biologics. 2021;15:115-132.

282. Scherer WF, Hurlbut HS. Nodamura virus from Japan: a new and unusual arbovirus resistant to diethyl ether and chloroform. Am J Epidemiol. 1967;86:271-285.

283. Schneemann A, Reddy V, Johnson JE. The structure and function of nodavirus particles: a paradigm for understanding chemical biology. Adv Virus Res. 1998;50:381-446.

284. Schneemann A, Zhong WD, Gallagher TM, Rueckert RR. Maturation cleavage required for infectivity of a nodavirus. J Virol. 1992;66:6728-6734.

285. Schultz KL, Friesen PD. Baculovirus DNA replication-specific expression factors trigger apoptosis and shutoff of host protein synthesis during infection. J Virol. 2009;83(21):11123-11132.

286. Schultz KL, Wetter JA, Fiore DC, Friesen PD. Transactivator IE1 is required for baculovirus early replication events that trigger apoptosis in permissive and nonpermissive cells. J Virol. 2009;83(1):262-272.

287. Selling BH, Allison RF, Kaesberg P. Genomic RNA of an insect virus directs synthesis of infectious virions in plants. Proc Natl Acad Sci U S A. 1990;87(1):434-438.

288. Seshagiri S, Miller LK. Baculovirus inhibitors of apoptosis (IAPs) block activation of Sf-caspase-1. Proc Natl Acad Sci U S A. 1997;94(25):13606-13611.

289. Seshagiri S, Vucic D, Lee J, Dixit VM. Baculovirus-based genetic screen for antiapoptotic genes identifies a novel IAP. J Biol Chem. 1999;274:36769-36773.

290. Settles EW, Friesen PD. Flock house virus induces apoptosis by depletion of *Drosophila* inhibitor-of-apoptosis protein DIAP1. J Virol. 2008;82(3):1378-1388.

291. Shi M, Lin XD, Tian JH, et al. Redefining the invertebrate RNA virosphere. Nature. 2016;540(7634):539-543.

292. Short JR, Knox C, Dorrington RA. Subcellular localization and live-cell imaging of the *Helicoverpa armigera* stunt virus replicase in mammalian and *Spodoptera frugiperda* cells. J Gen Virol. 2010;91(Pt 6):1514-1523.

293. Sinn PL, Burnight ER, Hickey MA, Blissard GW, McCray PB Jr. Persistent gene expression in mouse nasal epithelia following feline immunodeficiency virus-based vector gene transfer. J Virol. 2005;79(20):12818-12827.

294. Slack JM, Blissard GW. Identification of two independent transcriptional activation domains in the *Autographa californica* multicapsid nuclear polyhedrosis virus IE1 protein. J Virol. 1997;71(12):9579-9587.

295. Smith GE, Summers MD, Fraser MJ. Production of human beta interferon in insect cells infected with a baculovirus expression vector. Mol Cell Biol. 1983;3(12):2156-2165.

296. Sokal N, Nie Y, Willis LG, et al. Defining the roles of the baculovirus regulatory proteins IE0 and IE1 in genome replication and early gene transactivation. Virology. 2014;468-470:160-171.

297. Speir JA, Taylor DJ, Natarajan P, Pringle FM, Ball LA, Johnson JE. Evolution in action: N and C termini of subunits in related T = 4 viruses exchange roles as molecular switches. Structure. 2010;18(6):700-709.

298. Srinivasula SM, Ashwell JD. IAPs: what's in a name? Mol Cell. 2008;30(2):123-135.

299. Stapleford KA, Rapaport D, Miller DJ. Mitochondrion-enriched anionic phospholipids facilitate flock house virus RNA polymerase membrane association. J Virol. 2009;83(9):4498-4507.

300. Stewart TM, Huijskens I, Willis LG, Theilmann DA. The *Autographa californica* multiple nucleopolyhedrovirus ie0-ie1 gene complex is essential for wild-type virus replication, but either IE0 or IE1 can support virus growth. J Virol. 2005;79(8):4619-4629.

301. Stoltz DB, Vinson SB. Viruses and parasitism in insects. Adv Virus Res. 1979;24:125-171.

302. Stork NE. How many species of insects and other terrestrial arthropods are there on earth? Annu Rev Entomol. 2018;63:31-45.

303. Strand MR. Polydnaviruses. In: Asgari S, Johnson KN, eds. Insect Virology. Caister Academic Press; 2010:171-198.

304. Strand MR, Burke GR. Polydnaviruses: from discovery to current insights. Virology. 2015;479-480:393-402.

305. Strand MR, Burke GR. Polydnaviruses: evolution and Function. Curr Issues Mol Biol. 2020;34:163-182.

306. Strand MR, Pech LL. *Microplitis demolitor* polydnavirus induces apoptosis of a specific haemocyte morphotype in *Pseudoplusia includens*. J Gen Virol. 1995;76(Pt 2):283-291.

307. Suderman RJ, Pruijssers AJ, Strand MR. Protein tyrosine phosphatase-H2 from a polydnavirus induces apoptosis of insect cells. J Gen Virol. 2008;89(Pt 6):1411-1420.

308. Sun X. History and current status of development and use of viral insecticides in China. Viruses. 2015;7(1):306-319.

309. Szewczyk B, Hoyos-Carvajal L, Paluszek M, Skrzecz I, Lobo de Souza M. Baculoviruses—re-emerging biopesticides. Biotechnol Adv. 2006;24(2):143-160.

310. Taggart DJ. The baculovirus transcriptional regulator IE1 acts as an origin-binding protein to promote virus genome replication. *PhD Thesis, Department of Biochemistry, University of Wisconsin-Madison*. 2011.

311. Taggart DJ, Mitchell JK, Friesen PD. A conserved N-terminal domain mediates required DNA replication activities and phosphorylation of the transcriptional activator IE1 of *Autographa californica* multicapsid nucleopolyhedrovirus. J Virol. 2012;86(12):6575-6585.

312. Tan Y, Bideshi DK, Johnson JJ, Bigot Y, Federici BA. Proteomic analysis of the *Spodoptera frugiperda* ascovirus 1a virion reveals 21 proteins. J Gen Virol. 2009;90(Pt 2):359-365.

313. Tang L, Johnson KN, Ball LA, Lin T, Yeager M, Johnson JE. The structure of pariacoto virus reveals a dodecahedral cage of duplex RNA. Nat Struct Biol. 2001;8(1):77-83.

314. Taning CNT, Christiaens O, Li X, et al. Engineered flock house virus for targeted gene suppression through RNAi in fruit flies (*Drosophila melanogaster*) in Vitro and in Vivo. Front Physiol. 2018;9:805.

315. Tate J, Liljas L, Scotti P, Christian P, Lin TW, Johnson JE. The crystal structure of cricket paralysis virus: the first view of a new virus family. Nat Struct Biol. 1999;6(8):765-774.

316. Theilmann DA, Blissard GW, Bonning B, et al. Baculoviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, eds. Virus Taxonomy, 8th Report of the International Committee on Taxonomy of Viruses. Elsevier; 2005.

317. Theilmann DA, Stewart S. Identification and characterization of the IE-1 gene of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. Virology. 1991;180(2):492-508.

318. Theilmann DA, Stewart S. Molecular analysis of the trans-activating IE-2 gene of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. Virology. 1992;187(1):84-96.

319. Theze J, Bezier A, Periquet G, Drezen JM, Herniou EA. Paleozoic origin of insect large dsDNA viruses. Proc Natl Acad Sci U S A. 2011;108(38):15931-15935.

320. Thiem SM. Baculovirus genes affecting host function. In Vitro Cell Dev Biol Anim. 2009;45(3-4):111-126.

321. Thiem SM, Miller LK. Identification, sequence, and transcriptional mapping of the major capsid protein gene of the baculovirus *Autographa californica* nuclear polyhedrosis virus. J Virol. 1989;63(5):2008-2018.

322. Thoetkiattikul H, Beck MH, Strand MR. Inhibitor kappaB-like proteins from a polydnavirus inhibit NF-kappaB activation and suppress the insect immune response. Proc Natl Acad Sci U S A. 2005;102(32):11426-11431.

323. Tihova M, Dryden KA, Le TV, et al. Nodavirus coat protein imposes dodecahedral RNA structure independent of nucleotide sequence and length. J Virol. 2004;78(6):2897-2905.

324. Tomalski MD, Wu JG, Miller LK. The location, sequence, transcription and regulation of a baculovirus DNA polymerase gene. Virology. 1988;167(2):591-600.

325. Unchwaniwala N, Ahlquist P. Coronavirus dons a new crown. Science. 2020;369(6509):1306-1307.

326. Unchwaniwala N, Zhan H, den Boon JA, Ahlquist P. Cryo-electron microscopy of nodavirus RNA replication organelles illuminates positive-strand RNA virus genome replication. Curr Opin Virol. 2021;51:74-79.

327. van Oers MM. Opportunities and challenges for the baculovirus expression system. J Invertebr Pathol. 2011;107(suppl):S3-S15.

328. Van Wynsberghe PM, Ahlquist P. 5′ cis elements direct nodavirus RNA1 recruitment to mitochondrial sites of replication complex formation. J Virol. 2009;83(7):2976-2988.

329. Vanarsdall AL, Okano K, Rohrmann GF. Characterization of a baculovirus with a deletion of vlf-1. Virology. 2004;326(1):191-201.

330. Vanarsdall AL, Okano K, Rohrmann GF. Characterization of the replication of a baculovirus mutant lacking the DNA polymerase gene. Virology. 2005;331(1):175-180.

331. Vandergaast R, Mitchell JK, Byers NM, Friesen PD. Insect inhibitor-of-apoptosis (IAP) proteins are negatively regulated by signal-induced N-terminal degrons absent within viral IAP proteins. J Virol. 2015;89(8):4481-4493.

332. Vandergaast R, Schultz KL, Cerio RJ, Friesen PD. Active depletion of host cell inhibitor-of-apoptosis proteins triggers apoptosis upon baculovirus DNA replication. J Virol. 2011;85(16):8348-8358.

333. Ventor PA, Jovel J, Schneemann A. Insect nodaviruses. In: Asgari S, Johnson KN, eds. Insect Virology. Caister Academic Press; 2010:251-282.

334. Vialard JE, Richardson CD. The 1,629-nucleotide open reading frame located downstream of the *Autographa californica* nuclear polyhedrosis virus polyhedrin gene encodes a nucleocapsid-associated phosphoprotein. J Virol. 1993;67(10):5859-5866.

335. Volkman LE, Goldsmith PA, Hess RT, Faulkner P. Neutralization of budded *Autographa californica* NPV by a monoclonal antibody: identification of the target antigen. Virology. 1984;133:354-362.

336. Volkoff AN, Jouan V, Urbach S, et al. Analysis of virion structural components reveals vestiges of the ancestral ichnovirus genome. PLoS Pathog. 2010;6(5):e1000923.

337. Vucic D, Fairbrother WJ. The inhibitor of apoptosis proteins as therapeutic targets in cancer. Clin Cancer Res. 2007;13(20):5995-6000.

338. Vucic D, Kaiser WJ, Harvey AJ, Miller LK. Inhibition of reaper-induced apoptosis by interaction with inhibitor of apoptosis proteins (IAPs). Proc Natl Acad Sci U S A. 1997;94(19):10183-10188.

339. Vucic D, Kaiser WJ, Miller LK. Inhibitor of apoptosis proteins physically interact with and block apoptosis induced by *Drosophila* proteins HID and GRIM. Mol Cell Biol. 1998;18(6):3300-3309.

340. Walukiewicz HE, Johnson JE, Schneemann A. Morphological changes in the T=3 capsid of Flock House virus during cell entry. J Virol. 2006;80(2):615-622.

341. Wang M, Hu Z. Advances in Molecular Biology of Baculoviruses. Curr Issues Mol Biol. 2020;34:183-214.

342. Wang M, Tuladhar E, Shen S, et al. Specificity of baculovirus P6.9 basic DNA-binding proteins and critical role of the C terminus in virion formation. J Virol. 2010;84(17):8821-8828.

343. Wang R, Deng F, Hou D, et al. Proteomics of the *Autographa californica* nucleopolyhedrovirus budded virions. J Virol. 2010;84(14):7233-7242.

344. Wang S, Balasundaram G. Potential cancer gene therapy by baculoviral transduction. Curr Gene Ther. 2010;10(3):214-225.

345. Wang XH, Aliyari R, Li WX, et al. RNA interference directs innate immunity against viruses in adult *Drosophila*. Science. 2006;312(5772):452-454.

346. Wang Y, Bininda-Emonds OR, van Oers MM, Vlak JM, Jehle JA. The genome of *Oryctes rhinoceros* nudivirus provides novel insight into the evolution of nuclear arthropod-specific large circular double-stranded DNA viruses. Virus Genes. 2011;42(3):444-456.

347. Wang Y, Jehle JA. Nudiviruses and other large, double-stranded circular DNA viruses of invertebrates: new insights on an old topic. J Invertebr Pathol. 2009;101(3):187-193.

348. Warsaba R, Sadasivan J, Jan E. Dicistrovirus-host molecular interactions. Curr Issues Mol Biol. 2020;34:83-112.

349. Washburn JO, Chan EY, Volkman LE, Aumiller JJ, Jarvis DL. Early synthesis of budded virus envelope fusion protein GP64 enhances *Autographa californica* multicapsid nucleopolyhedrovirus virulence in orally infected *Heliothis virescens*. J Virol. 2003;77(1):280-290.

350. Webb BA, Strand MR, Dickey SE, et al. Polydnavirus genomes reflect their dual roles as mutualists and pathogens. Virology. 2006;347(1):160-174.

351. Webster CL, Longdon B, Lewis SH, Obbard DJ. Twenty-Five New Viruses Associated with the Drosophilidae (Diptera). Evol Bioinform Online. 2016;12(Suppl 2):13-25.

352. Weeks SA, Shield WP, Sahi C, Craig EA, Rospert S, Miller DJ. A targeted analysis of cellular chaperones reveals contrasting roles for heat shock protein 70 in flock house virus RNA replication. J Virol. 2010;84(1):330-339.

353. Weitzman MD, Lilley CE, Chaurushiya MS. Genomes in conflict: maintaining genome integrity during virus infection. Annu Rev Microbiol. 2010;64:61-81.

354. Williams GV, Rohel DZ, Kuzio J, Faulkner P. A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus *p10* gene function using insertion-deletion mutants. J Gen Virol. 1989;70(1):187-202.

355. Williams T, Bergoin M, van Oers MM. Diversity of large DNA viruses of invertebrates. J Invertebr Pathol. 2017;147:4-22.

356. Wilson ME, Consigli RA. Functions of a protein kinase activity associated with purified capsids of the granulosis virus infecting *Plodia interpunctella*. Virology. 1985;143:526-535.

357. Wilson ME, Miller LK. Changes in the nucleoprotein complexes of a baculovirus DNA during infection. Virology. 1986;151(2):315-328.

358. Windus LCE, Jones AM, Downes S, Walsh T, Knight K, Kinkema M. HearNPV susceptibility in Helicoverpa armigera and *Helicoverpa punctigera* strains resistant to Bt toxins Cry1Ac, Cry2Ab, and Vip3Aa. J Invertebr Pathol. 2021;183:107598.

359. Wright CW, Clem RJ. Sequence requirements for Hid binding and apoptosis regulation in the baculovirus inhibitor of apoptosis Op-IAP. Hid binds Op-IAP in a manner similar to Smac binding of XIAP. J Biol Chem. 2002;277(4):2454-2462.

360. Wright CW, Means JC, Penabaz T, Clem RJ. The baculovirus anti-apoptotic protein Op-IAP does not inhibit *Drosophila* caspases or apoptosis in *Drosophila* S2 cells and instead sensitizes S2 cells to virus-induced apoptosis. Virology. 2005;335(1):61-71.

361. Wu Y, Carstens EB. Initiation of baculovirus DNA replication: early promoter regions can function as infection-dependent replicating sequences in a plasmid-based replication assay. J Virol. 1996;70(10):6967-6972.

362. Wu YL, Wu CP, Lee ST, et al. The early gene hhi1 reactivates *Heliothis zea* nudivirus 1 in latently infected cells. J Virol. 2010;84(2):1057-1065.

363. Wu YL, Wu CP, Liu CYY, Hsu PW, Wu EC, Chao YC. A non-coding RNA of insect HzNV-1 virus establishes latent viral infection through microRNA. Sci Rep. 2011;1:60. doi: 10.1038/srep00060

364. Xu B, Yoo S, Guarino LA. Differential transcription of baculovirus late and very late promoters: fractionation of nuclear extracts by phosphocellulose chromatography. J Virol. 1995;69(5):2912-2917.

365. Xu G, Cirilli M, Huang Y, Rich RL, Myszka DG, Wu H. Covalent inhibition revealed by the crystal structure of the caspase-8/p35 complex. Nature. 2001;410(6827):494-497.

366. Yamagishi J, Burnett ED, Harwood SH, Blissard GW. The AcMNPV pp31 gene is not essential for productive AcMNPV replication or late gene transcription but appears to increase levels of most viral transcripts. Virology. 2007;365(1):34-47.

367. Yang S, Miller LK. Activation of baculovirus very late promoters by interaction with very late factor 1. J Virol. 1999;73(4):3404-3409.

368. Yoo S, Guarino LA. The *Autographa californica* nuclear polyhedrosis virus ie2 gene encodes transcriptional regulator. Virology. 1994;202(2):746-753.

369. Young JC, MacKinnon EA, Faulkner P. The architecture of the virogenic stroma in isolated nuclei of *Spodoptera frugiperda* cells in vitro infected by *Autographa californica* nuclear polyhedrosis virus. J Struct Biol. 1993;110:141-153.

370. Zaghloul HAH, Hice R, Arensburger P, Federici BA. Transcriptome analysis of the Spodoptera frugiperda Ascovirus in vivo provides insights into how its apoptosis inhibitors and caspase promote increased synthesis of viral vesicles and virion progeny. J Virol. 2017;91(23):e00874-17.

371. Zaghloul HAH, Hice RH, Arensburger P, Bideshi DK, Federici BA. Extended in vivo transcriptomes of two ascoviruses with different tissue tropisms reveal alternative mechanisms for enhancing virus reproduction in hemolymph. Sci Rep. 2021;11(1):16402.

372. Zhao S, He G, Yang Y, Liang C. Nucleocapsid assembly of Baculoviruses. Viruses. 2019;11(7):595.

373. Zhou J, Blissard GW. Identification of a GP64 subdomain involved in receptor binding by budded virions of the baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus. J Virol. 2008;82(9):4449-4460.

374. Zhou Q, Krebs JF, Snipas SJ, et al. Interaction of the baculovirus anti-apoptotic protein p35 with caspases. Specificity, kinetics, and characterization of the caspase/p35 complex. *Biochemistry* (*Mosc*). 1998;37(30):10757-10765.

375. Zlotnick A, Reddy VS, Dasgupta R, et al. Capsid assembly in a family of animal viruses primes an autoproteolytic maturation that depends on a single aspartic acid residue. J Biol Chem. 1994;269(18):13680-13684.

376. Zoog SJ, Bertin J, Friesen PD. Caspase inhibition by baculovirus P35 requires interaction between the reactive site loop and the beta-sheet core. J Biol Chem. 1999;274(37):25995-26002.

377. Zoog SJ, Schiller JJ, Wetter JA, Chejanovsky N, Friesen PD. Baculovirus apoptotic suppressor P49 is a substrate inhibitor of initiator caspases resistant to P35 in vivo. EMBO J. 2002;21(19):5130-5140.

CHAPTER 20

Viruses and Prions of Yeasts, Fungi, and Protists

Reed B. Wickner

Double-stranded RNA viruses

Totivirus type species L-A of *Saccharomyces cerevisiae*

Partitiviridae

Reovirus of Cryphonectria

Single-stranded RNA viruses

Viruses reducing virulence of chestnut blight fungus

Negative strand RNA mycoviruses

Narnaviridae 20S RNA, 23S RNA, and mitoviruses

Hadaka [naked] viruses and *yadokari [home-borrowing] viruses*

Chromosomal genes affecting mycoviruses

Single-stranded DNA gemini-like mycovirus

Retroviruses (retroelements)

Retroviruses, retrotransposons, retroposons, and retrointrons

Structure of Tys

Replication cycle of *Saccharomyces cerevisiae* Ty elements

Expression of Tys

Schizosaccharomyces pombe retroelements

Candida albicans plasmid retrotransposon and line elements

Prions of *Saccharomyces* and *Podospora*

[URE3]—a transmissible amyloidosis of Ure2p

[PSI+]—a transmissible amyloidosis of Sup35p

[PIN+]—a prion that generates other prions

[Het-s]—a prion of podospora responsible for a normal function

Prion variants

Structures of yeast prion amyloids explain variant propagation

HET-s prion amyloid structure

[URE3] and [PSI+] are diseases of yeast

Chaperone involvement in prion propagation

Antiprion systems in *Saccharomyces cerevisiae*

Perspectives

Acknowledgments

Prions, retroviruses, double-stranded RNA (dsRNA) viruses, (+) and (−) single-stranded RNA (ssRNA) viruses and single- and double-stranded DNA (ssDNA and dsDNA) viruses have all been found in simple eukaryote hosts, and the experimental advantages of these systems have made some of them the most thoroughly characterized of their classes. This is an overview of these infectious entities and their unique aspects, with some emphasis on recent developments. We focus on the most-studied virus of each group because of space limitations (Table 20.1).

TABLE 20.1 Representative viruses of simple eukaryotes

Virus	Family	Typical	Features
dsRNA viruses	*Totiviridae*	L-A of *S cerevisiae*	One segment, Gag-Pol, "T = 2" icosahedral, killer toxin satellites
	Partitiviridae	*Atkinsonella hypoxylon virus* (AhV) [291]	2 (or 3) segments separately encapsidated, "T = 2" capsid[390]
	Mycoreoviridae	MRV1 *C parasitica*	11 segments, similar to *Coltivirus*, hypovirulence
(+)ssRNA viruses	*Hypoviridae*	CHV1-EP713 of *Cryphonectria parasitica*	1 segment, in vesicles, no capsid, hypovirulence, two proteases, anti-RNAi
	Narnaviridae	20S, 23S RNAs of *S cerevisiae*	No coat, RDRP encoded, similar to Qβ
	Mitoviridae	CpMV1 of *C parasitica*	No coat, RDRP encoded, similar to Qβ, UGA = Trp, intra-mitochondrial
(–) ssRNA viruses	*Mymonaviridae* (mononegavirus)	SsNSRV-1 of *Sclerotinia sclerotiorum*	filamentous particles, 1 segment, 6 ORFs [248]
ssDNA viruses	*Genomoviridae* (*Gemini*-like)	SsHADV-1 of *S*	Infectious for fungus and insect
Retro-elements	Retrotransposon (Ortervirales)	Ty1, Ty3 of *S cerevisiae*	LTR retrotransposons, Ty1 in *copia* group, Ty3 in *gypsy* group
	Retroposon	Zorro3 of *Candida albicans*	no LTR, LINE-like
	Retrointron	introns aI1 and aI2 of *COX1* mitochondrial gene of *S cerevisiae*	no LTR, retro-mobile intron

dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; ssDNA, single-stranded DNA; LTR, long terminal repeat. Based on amino acid sequence homology and gene order, retrotransposons may be divided into those similar to the *copia* element or the *gypsy* element of *Drosophila*.

Nearly all of the viruses, prions, and retrotransposons (or retroviruses) of yeast and fungi have wholly intracellular life cycles. These viruses have forsaken the extracellular route of transmission, being passed from cell to cell either vertically or by cytoplasmic mixing, such as occurs in mating, or in hyphal anastomosis (fusion) of filamentous fungi. They resemble, in this regard, the plant cryptoviruses and the intracisternal A type particles (retroelements) and LINEs (retroposons) of mammals. The high frequency of mating and hyphal fusion of fungi in nature makes an extracellular phase dispensable for these viruses. Indeed, many wild *Saccharomyces cerevisiae* carry the RNA viruses, and it is difficult to find strains of *S cerevisiae* that lack the Ty retrotransposons. Nor is the direct cell-to-cell route of infection completely neglected by mammalian viruses—those of the herpes group and human immunodeficiency virus being prime examples. However, a *Totivirus* of the human parasite *Giardia lamblia* and a Gemini-like ssDNA virus of the plant pathogenic fungus *Sclerotinia sclerotiorum* are each infectious by an extracellular route for their respective hosts (see below).

Recently, sequences of many yeast genomes have revealed occasional (diverged) copies of the yeast L-A viral and M_2 killer toxin genomes.[376] Interestingly, the integrated viral sequences from distantly related yeasts were less diverged than their hosts, indicating horizontal transfer.[376] The means by which this transfer occurs is not yet clear.

The largely intracellular mode of transmission means that viruses must balance the need to spread and propagate against consideration for the viability of their host. This is clearly seen in the Ty elements, which tend to target sites that can tolerate an insertion. Ty1 limits its transposition by making a fragment of Gag that inhibits particle assembly and other functions of Gag.[162,334] The Ty elements, particularly Ty3, regulate their transcription such that most transposition occurs on mating.[219] This design optimizes the chances of infecting the potentially unoccupied genome of the mating partner. Furthermore, the fact that most of these elements are widespread means that the hosts have been selected for the ability to protect themselves. The copy numbers of several of the RNA replicons (L-A dsRNA, L-BC dsRNA, and 20S RNA) are repressed by the host Ski proteins acting in translation,[261,343] messenger RNA (mRNA) stability,[198] and positive strand stability.[143] The Nuc1 endonuclease also protects the host from the dsRNA viruses, particularly during meiosis.[155] The host blocks Ty1 retrotransposition at several levels: translation, virus particle and DNA stability, structure of the chromatin target (eg, see Refs.[28,74,191,266]).

Most of the viruses and prions discussed in this chapter can best be viewed as selfish RNA or DNA (or selfish protein); however, the killer satellite dsRNAs clearly benefit their hosts (and promote the L-A helper virus) by allowing them to kill competitors. The [Het-s] prion of *Podospora* is unique among prions described to date in being necessary for a normal cellular function—namely, vegetative (heterokaryon) incompatibility that is expected to block horizontal transfer of viruses and transposons (see below).[81]

Yeast and fungal prions have led the way in proving the prion concept, defining the structure of prion amyloids, and in relating those structures to prion biology and heritability (reviewed in Ref.[404]). Moreover, the study of yeast prions has explored myriad chaperones and other host components affecting prion generation and propagation (reviewed in Ref.[27,263]). Host antiprion systems dramatically limit the frequency of prions arising and cure many that do arise; their manipulation may provide a means of treating human amyloid/prion diseases the way the immune systems are manipulated to deal with bacterial and viral diseases (reviewed in Ref.[401]).

It is striking that many of the viruses and retroelements discussed use some form of ribosomal frameshifting to make a "Gag-Pol" fusion protein. The fusion of the capsid protein and the RNA polymerase provides packaging of the polymerase and, by the polymerase binding to the (+) RNA strand, a means of packaging the genome (see below). Read-through of stop codons could do the same thing.

The use of *S cerevisiae* as a model host for viruses of plants, mammals, and insects promises to facilitate the understanding of these viruses[282,283,306] but will not be dealt with here.

Double-Stranded RNA Viruses

Fungal dsRNA viruses were first detected in 1948 as “La France” disease of cultivated mushrooms in Pennsylvania, and its study led to the first description of fungal viruses in 1962. The antiviral agents helenine and statolon, discovered in *Penicillium* in the 1950s, later proved to be fungal virus dsRNA that was inducing interferon production. The dsRNA viruses found in many fungal species include the single-segment *Totiviridae*, the oligosegmented *Partitiviridae*, and the 10-11 segmented Mycoreovirus.

Totivirus Type Species L-A of *Saccharomyces cerevisiae*

Some strains of *S cerevisiae* secrete a protein toxin that is lethal to other strains but to which they are themselves immune (Fig. 20.1). This killer character of some strains is inherited as a non-mendelian genetic element, and its study led to the discovery of the L-A dsRNA virus and its separately encapsidated satellites M_1, M_2, M_{28}, and so on, each M encoding a different toxin-immunity specificity.

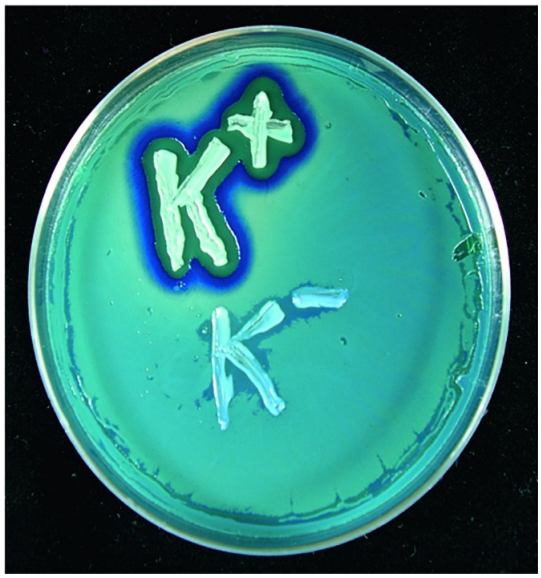

FIGURE 20.1 **The killer phenomenon of *Saccharomyces cerevisiae*.** A sensitive strain of yeast was spread as a lawn, and streaks of a killer strain (*above*) or a nonkiller strain (*below*) were applied. After 2 days of incubation, the lawn of the sensitive strain has not grown in a zone around the killer strain. The secreted protein killer toxin, and immunity to the toxin, are encoded by M_1, a satellite double-stranded RNA (dsRNA) of the L-A dsRNA virus.

L-A Virion Structure

The icosahedral structures of L-A and all Totiviruses examined to date combine T = 1 icosahedral symmetry with 120 coat protein molecules per particle[134,284] (Fig. 20.2). This same symmetry is found in the cores of most other dsRNA viruses examined, including partitiviruses, reovirus, rotavirus, bluetongue virus, aquareovirus, and bacteriophage φ6 (reviewed in Ref.[265] and see Chapters 3 and 21). Because sequence data suggest that different dsRNA virus groups each arose independently from different ssRNA viruses,[223] rather than one being descended from each other, their common structure suggests that there is something about this structure that is well adapted to the intraviral replication pattern of dsRNA viruses.

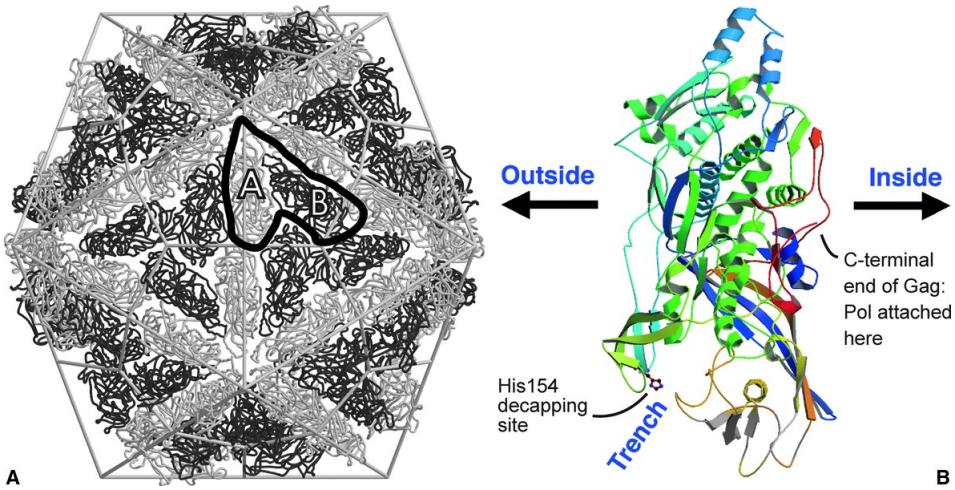

FIGURE 20.2 **L-A double-stranded RNA virus capsid at 3.4-Å resolution from X-ray crystallography.** **A**. Overall organization of the L-A capsid. Identical subunits occupy two different environments, with “A” subunits (*light blue*) contacting the two- and fivefold axes and “B” subunits contacting the threefold axes. **B**. A single A subunit is shown with the trench on the outside surface including

His154, the site of the decapping activity. (Adapted from Naitow H, Tang J, Canady M, et al. L-A virus at 3.4 A resolution reveals particle architecture and mRNA decapping mechanism. *Nat, Struct Biol*. 2002;9(10):725-728; Adapted from Tang J, Naitow H, Gardner NA, et al. The structural basis of recognition and removal of cellular mRNA 7-methyl G 'caps' by a viral capsid protein: a unique viral response to host defense. *J Mol Recogn*it. 2005;18(2):158-168.)

The combination of 120 subunits and T = 1 icosahedral symmetry implies that individual coat protein monomers can be in either of two environments. This expectation has been verified as subtly different morphology of the nonequivalent Gag protomers[284] (see Fig. 20.2). The packing of the dsRNA within the particles is less dense than is typical for dsDNA viruses, possibly reflecting the fact (see later discussion) that in both replication and transcription, the genome must translocate sequentially past the RNA-dependent RNA polymerase immobilized on the particle wall, and this requires more space than is required by the static DNA genomes.[46] The L-A virions also have 10- to 15-Å pores, which allow entry of nucleotides and exit of positive-strand transcripts but do not allow the dsRNA genome to exit or degradative enzymes to enter.[284] A trench on the outer virion surface contains His154 of Gag, the site of the mRNA decapping activity, and several nearby residues essential for this activity have been identified.[375]

L-A Genome Structure

The L-A positive strand has two overlapping open reading frames (ORFs)[195] (Fig. 20.3A). They are *gag*, encoding the 76-kd major coat protein, and *pol*, encoding the 100-kd Pol domain of the Gag-Pol fusion protein.[149,195] The pol ORF includes the consensus amino acid sequence patterns typical of viral RNA-dependent RNA polymerases. Three *in vitro* ssRNA binding domains have also been localized to Pol,[149,315] the central one cryptic unless an adjacent inhibitory region is deleted (see Fig. 20.3A). Pol residues 67-213 are necessary for packaging of the viral positive strands.[147,315] Neither 5′ cap structure nor 3′ poly(A) has been found on either strand of genomic L-A dsRNA or M_1 dsRNA.[144] The genome has 5′ diphosphates on both strands [144]and an uncoded base (A or G) at each 3′ end.[41]

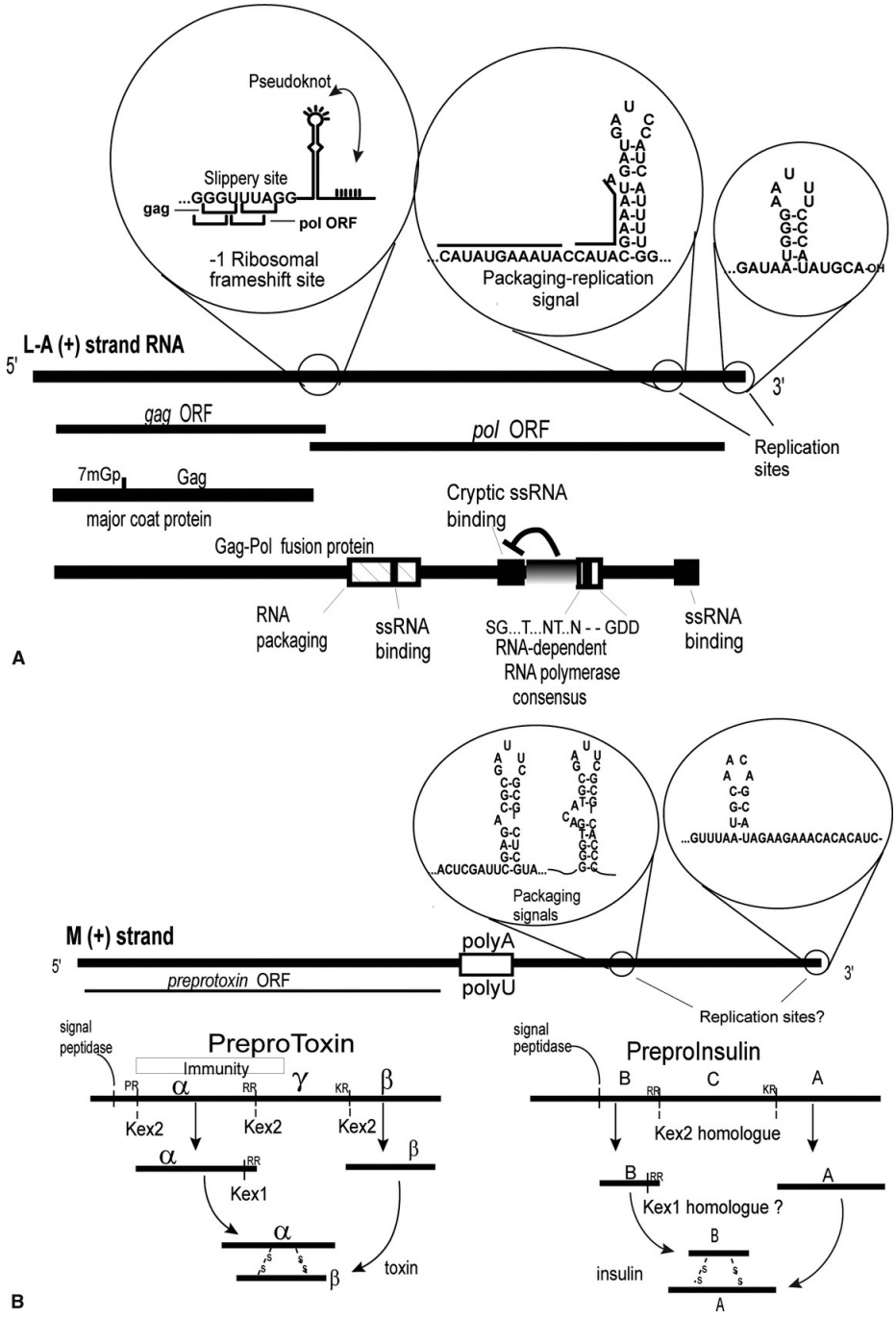

FIGURE 20.3 **A**. Sites and encoded proteins of the L-A positive strand. The sites responsible for -1 ribosomal frameshifting, positive single-stranded RNA packaging, and replication are shown. The packaging signals include direct repeat sequences (shown by an overline for L-A). The pseudoknot makes the ribosome pause over the slippery site, where the transfer RNAs can unpair from the messenger RNA and re-pair in the -1 frame with correct base pairing of the nonwobble bases. Functional domains in the Pol region are also indicated. 7mGp indicates the cap-binding site at His154 of Gag. **B**. Coding and *cis* sites of M1 positive strand. The analogous and homologous processing of the K1 preprotoxin encoded by M1 is compared to that of preproinsulin.

The M dsRNAs each encode, in their 5′ portion, a preprotoxin protein[36,103,251,342] (Fig. 20.3B). The M_1 positive strand has an internal encoded poly(A) region whose length shows frequent clonal variation, presumably owing to transcriptase stuttering, and a substantial 3′ region that encodes no protein but contains essential *cis* sites.[131,145,165,331,350] Sites on the L-A and M_1 positive strands necessary for packaging and negative-strand synthesis have been determined (see later discussion and Fig. 20.3), and some limits have been set on the possible transcription signals.

Stages in the Replication Cycle

The L-A replication cycle and the closely related cycle of its satellites are shown in Figure 20.4.[134,146] L-A dsRNA-containing viral particles synthesize positive ssRNA in a conservative reaction,[146] and these new positive strands are then extruded from the particle. There, they serve as mRNA for the production of the Gag and the Gag-Pol fusion proteins. These proteins then assemble with a viral positive strand to form new particles. The newly assembled viral particles carry out the synthesis of negative strands on the positive-strand template to form dsRNA and

complete the cycle (see Cap-snatching below).

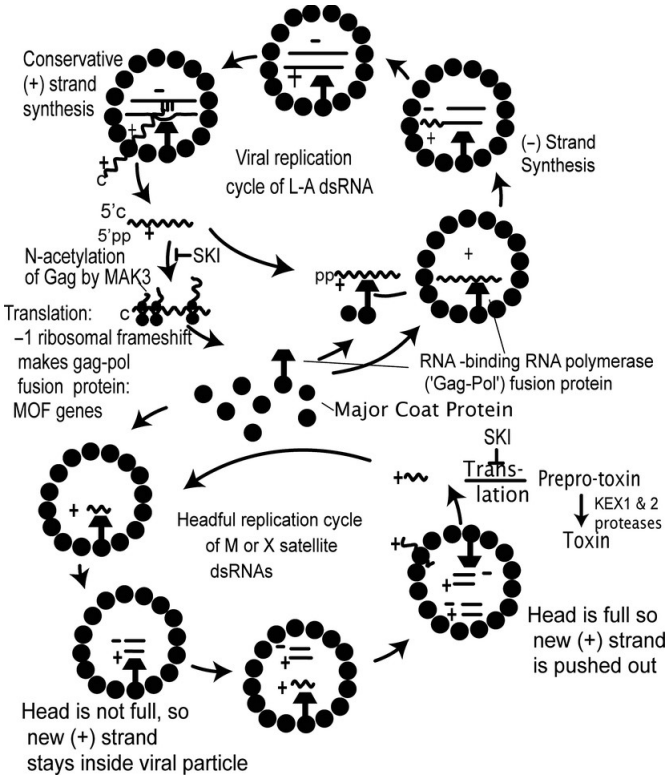

FIGURE 20.4 **Replication cycles of L-A virus and its satellite double-stranded RNAs (dsRNAs), M and X**. The packaging region of the Pol domain of Gag-Pol binds to the packaging site of positive strands as the Gag domain associates with other Gag molecules. This ensures packaging of viral positive strands.[147,149] L-A particles have only one dsRNA molecule per particle. However, the smaller M or X dsRNAs replicate within the viral coat until they fill the head.[134,136] The (+) strand transcripts that are capped by Gag are translated, while those with an uncapped 5′ diphosphate end are packaged.[144]

In addition to their appropriating viral proteins from L-A, the replication cycle of the satellite dsRNAs, M_1, S (deletion mutants of M_1), and X (a 530 base pair [bp] deletion mutant of L-A) are similar to that of L-A itself except that they replicate more than once within the viral particle until it is full (see Fig. 20.4). Only a single positive ssRNA is packaged per particle[145]; thus, L-A and M are separately encapsidated. Because the particle capacity is determined by the structure of Gag to be sufficient to accommodate one full-length L-A dsRNA molecule, positive-strand transcripts of the smaller M and X dsRNAs are often retained within the particles, where they are converted to new dsRNA molecules. All new positive strands are extruded from the full particles.[134,136] This headful replication mechanism contrasts with the headful packaging mechanism of many DNA bacteriophage. This implies that extrusion of the transcripts is a mechanical consequence of the head being full rather than an active process. Both positive and negative strands are synthesized within the viral particles, but at different points in the cycle; therefore, the replication is conservative, intraviral, and asynchronous, and it fills the capsid (headful mechanism).

Transcription Reaction (Positive-Strand Synthesis)

As in the *Reoviridae*, the transcription reaction for L-A is conservative, resulting in the overall process of viral replication being conservative. However, in *Aspergillus foetidus* slow virus and *Penicillium stoloniferum* slow virus, dsRNA transcription is semiconservative. The difference between conservative and semiconservative reactions concerns whether or not there is re-pairing of the template negative strand with the parental positive strand that was (in either case) displaced during the synthesis.

Treatment of L-A virus particles with very low ionic strength results in their rupture and release of the dsRNA. These opened empty particles can be reisolated free of RNA and carry out a dsRNA template-dependent reaction that is, like the *in vivo* reaction, conservative.[151] This reaction is highly template specific, using only L-A, M, and X dsRNAs (all physiologic templates) but not L-BC, p6, or rotavirus dsRNAs.[151] Because X dsRNA retains only 25 bp of the end of L-A from which the transcription reaction starts,[136] the signal recognized by the transcriptase is most likely within this region, perhaps within the terminal 6 bp that are in common among L-A, M_1, and M_2. The template-dependent transcription reaction requires very high concentrations of polyethylene glycol (20%), suggesting that the transcriptase has a low affinity for the dsRNA template.[151] Because the dsRNA is normally formed within and stays inside the viral particles, the RNA polymerase sees a very high effective concentration and need not have a particularly high affinity.

Translation

The translation apparatus is a prime battleground for the fight between an RNA virus and its host. For example, interferon acts primarily by blocking viral translation. Picornaviruses cleave a cap-binding protein inactivating host translation, and influenza virus steals caps from cellular mRNAs (Chapter XX). RNAi also blocks translation of target mRNAs or promotes their degradation. Studies of the L-A virus and its satellites likewise suggest that translation of viral proteins is a critical event determining the balance between virus and host.

Ribosomal frameshifting

The *gag* and *pol* ORFs of L-A overlap by 130 nucleotides, and the Pol protein is expressed only as a fusion protein with the major coat protein, Gag[149,195] (see Fig. 20.3A). The mechanism of formation of this Gag-Pol fusion protein is a −1 ribosomal frameshift,[105] very similar to those described in many retroviruses, coronaviruses, and several plant viruses (reviewed in Ref.[9]).

The frequency of −1 ribosomal frameshifts is determined by the "slippery site," a sequence of the form X XXY YYZ, where the *gag* reading frame is shown, and by a downstream pseudoknot (see Fig. 20.3A). The slippery site allows the tRNAs reading XXY and YYZ to move back one base on the mRNA and still have their nonwobble bases correctly paired.[196] X can be any base, although Y can be only A or U, probably because the frequency with which unpairing of the tRNAs from the 0 frame codons (XXY and YYZ) is also important, and because A site pairing is stronger than P site pairing.[42,105] Z can be any base but G, suggesting that specific tRNAs are more able to frameshift than others.[50] The RNA pseudoknot, just downstream of the slippery site, slows ribosomal movement at this point.[42,105] The location of this secondary structure is particularly critical,[42] and a pseudoknot is far more effective in promoting frameshifting than a simple stem loop of the same overall melting energy. The pseudoknot, more than the simple stem loop, should halt melting of the stem at a unique point because the rotation of the stem is limited by the downstream stem. This precisely positions the ribosome with the slippery site in the A and P sites.[303]

The efficiency of −1 frameshifting, and thus the ratio of Gag-Pol fusion protein to Gag protein produced, is critical for viral propagation,[107] as is the efficiency of +1 frameshifting for Ty1 retrotransposition frequency (see later discussion). A twofold change away from L-A's normal 1.9% efficiency results in failure to propagate the M_1 satellite dsRNA. L-A propagation is less sensitive; however, the antibiotics sparsomycin, which increases ribosomal frameshifting, and anisomycin, which has the opposite effect, can cure L-A.[106] The Gag-Pol to Gag ratio is likely to be important for viral assembly. Excess Gag-Pol (high frameshift efficiency) may result in starting too many particles and winding up with too little Gag to complete any of them. In contrast, excess Gag might result in particles closing before the packaging domain of Pol has had a chance to find a viral positive strand.[106]

Host genes affecting the efficiency of −1 frameshifting are referred to as *MOF* (maintenance of frame).[107] The differential effects of specific host mutations on frameshifting at specific slippery sites suggests that drugs similarly affecting frameshifting might target specific viruses. *MOF9* is the 5S ribosomal RNA (rRNA), showing a role for this 60S subunit component in maintenance of reading frame.[108] *MOF6* is *RPD3* encoding a histone deacetylase; the *mof6-1* allele affects rRNA processing in such a way that ribosomal frameshifting is increased.[276] Ribosomal frameshifting is also used in some mammalian genes, such as Edr (embryonal carcinoma differentiation regulated),[353] and various yeast genes affected by a frameshifting site have been identified.[25] Whereas viral ribosomal frameshifting produces two viral proteins in a desired ratio, that of yeast cellular genes increases mRNA instability by diverting translating ribosomes to termination codons in another frame and inducing nonsense-mediated decay.[25] Viral sequences suggest that many Totiviruses use −1 ribosomal frameshifting to make fusion proteins, but a −2 shift occurs in a *Trichomonas vaginalis* Totivirus[294] and a +1 frameshift appears in a dsRNA Amalgavirus infecting the grasshopper pathogen fungus (microsporidian) *Antonospora locusta*.[308] The 190S virus of *Helminthosporium victoriae* (Hv190SV) is a totivirus that makes separate coat protein and RNA polymerase by a re-initiation: the termination codon for the coat protein overlaps with the initiation codon for the RNA polymerase, the sequence being...GGA CA**A TG A** GT G....[193]

60S subunits—critical to viral propagation

Mutations in any of 20 chromosomal genes (called *MAK* [maintenance of killer]) that result in diminished levels of free 60S ribosomal subunits also produce loss of M dsRNA and decreased copy number of L-A dsRNA.[120,292] Mutations diminishing the supply of free 40S subunits had no such effect.[292] The deficiency of 60S subunits results in selectively decreased rates of translation of mRNAs which, like L-A mRNA, lack 3′ poly(A).[120] These *mak* mutations are suppressed by the *ski* mutations that derepress translation of non-poly(A) mRNAs.[261]

Cap-snatching by Gag separates (+) strands for translation from those for replication

The L-A and L-BC Gag proteins each covalently bind the 5′ cap from any RNA *in vitro*.[30] L-A Gag covalently attaches ^{7m}GMP from the cap to His154 in a reaction that requires only Mg^{2+}. L-A virions can then transfer the cap structure to nascent L-A (and presumably M) mRNAs as they are synthesized.[142,143] Modification of His154 destroys Gag's ability to covalently bind cap, resulting in loss of killer toxin expression from M_1.[31,142] However, the (+) strands of viral dsRNA do not have detectable cap; both (+) and (−) strands have mostly diphosphate 5′ ends with a minority of 5′ triphosphate ends.[144] Using opened empty viral particles that can carry out *in vitro* transcription with specificity for L-A or M related templates,[151] Fujimura and Esteban have shown that only templates with a (+) strand 5′ diphosphate can serve as transcription templates, but the (−) strand synthesis reaction is indifferent to this modification.[144] It is suggested that not all transcripts emerging from the viral particles are capped, and that capped transcripts will be translated, and those that are uncapped will be packaged and propagate.[144] Influenza virus polymerase uses 5′ cap structures with 10-13 nucleotides that it cuts from a host RNA to prime viral mRNA synthesis, in contrast to the L-A cap-snatching, which appears to be added after the mRNA 5′ end has been synthesized.

The *SKI1/XRN1* gene encodes a 5′-3′ exoribonuclease, specific for uncapped RNAs that is responsible for the major mRNA decay pathway.[190] Overexpression of Ski1p can cure the L-A virus.[133] Mutation of the *SKI1/XRN1* gene restores expression of killer toxin despite mutation of His154 of Gag,[261] indicating that cells can translate an mRNA lacking both cap and poly(A).

Antiviral Systems

Ski2 antiviral system blocks translation of viral [non-poly(A)] messenger RNA

The sole essential function of *SKI2, 3, 7*, and *8* of *S cerevisiae* is the repression of viral copy number.[26,155,314,357,407] The SKI proteins repress three unrelated viral systems: L-A and its satellites, L-BC (a dsRNA virus), and the ssRNA replicon referred to as 20S RNA.[15,136,267,310,383] The *ski2*Δ, *ski3*Δ, *ski7*Δ, and *ski8*Δ mutants are each cold-sensitive for growth if they carry M_1 dsRNA,[26] and *ski2*Δ strains can become sick or die at

any temperature if, in addition, the [D] cytoplasmic element is present.[135] The molecular nature of [D] is as yet unknown.

Detailed studies of *SKI2* suggested that the system acts by limiting translation of viral mRNA.[261,407] None of these cytoplasmic replicons has 3′ poly(A) structures, and electroporation of reporter mRNAs into spheroplasts shows that the Ski2, Ski3, Ski7, and Ski8 proteins inhibit the translation of mRNA specifically if it lacks a 3′ poly(A) structure.[26,261] Both the initial rates and the duration of translation are affected by the Ski proteins, suggesting that both translation and mRNA stability are affected.

Ski2p is a helicase conserved from yeast to man,[407] Ski3p is a TPR repeat protein,[314] and Ski8p has a repeat first identified in β-transducin.[357] Ski2p, Ski3p, and Ski8p form a cytoplasmic complex[43] that is bound to ribosomes[341] where it extracts translation-arrested mRNAs from the ribosome.[429] These mRNAs are transferred for degradation to a complex of 3′ to 5′ exoribonucleases called the RNA exosome[198] (reviewed by Ref.[211]). The removal of stalled mRNAs from ribosomes and its transfer to RNA exosomes may explain the combined effects on translation and degradation of non-poly(A) mRNAs.

SLH1 is a yeast homolog of *SKI2*, and like *ski2Δ*, *slh1*Δ has elevated L-A copy number.[260] Like the Ski2-3-8 complex, the Slh1 helicase is also involved in dissociating stalled ribosomes.[269] The *ski2Δ slh1*Δ double mutants show dramatically increased L-A, L-BC, and M_1 dsRNA copy numbers but grow normally in the absence of the L-A and M_1 viruses.[343] Remarkably, the *ski2Δ slh1*Δ cells show the same *in vivo* translation kinetics of Cap+ non-poly(A) mRNA as they do for Cap+ poly(A)+ mRNA, despite the presence of normal amounts of competing poly(A)+ mRNA.[343] The translation apparatus seems indifferent to the 3′ poly(A) structure except for the action of the Ski proteins and Slh1. This suggests that one role of the eukaryote 3′ poly(A) is to allow cells to distinguish their own mRNAs from those of invading viral genomes.

Nuc1 and Ski3 Block Viral Lethality in Meiosis

Mutation of either *POR1*, encoding the major mitochondrial outer membrane porin, or *NUC1*, encoding the major mitochondrial nuclease, results in derepression of L-A copy number.[104,250] [A similar effect results from mutation of *scs1/lcb2*,[163] encoding a subunit of serine palmitoyltransferase.]

Recently, Gao et al. have found that Nuc1 and Ski3 cooperate to limit viral pathogenesis particularly in the meiosis-sporulation process.[155] Sporulation of *ski3Δ/ski3Δ* M_1^+ diploids produces only dead spores, although the parent diploids are quite healthy. In contrast, *ski3Δ/ski3Δ* diploids without M dsRNA produce all viable spores and *kex1Δ/kex1Δ ski3Δ/ski3Δ* M_1^+ diploids produce mostly viable healthy segregants. Kex1 is involved in M protoxin maturation, suggesting that toxin overproduction is killing the sporulating *ski3Δ/ski3Δ* M_1^+ cells. Examination of sporulating cells shows that while normal cells have little or none of the mature (secreted) killer toxin, *nuc1Δ/nuc1Δ* K_1^+ and *ski3Δ/ski3Δ* K_1^+ cells accumulate a great deal of toxin. If M_1 is present *during meiosis and sporulation*, *nuc1Δ* and *ski3Δ* are synthetic lethal. Nuc1 is normally confined to the mitochondria but partially enters the cytoplasm during sporulation. A *por1Δ* or *por2Δ* mutant does not release Nuc1 into the cytoplasm during sporulation and so behaves like a *nuc1Δ* strain in causing lethality if M_1 is present and the cells are *ski3Δ*.[155]

While the previous paragraph describes pathology due to derepressed M_1, probably due in each case to the toxin, derepressed L-A also is toxic to cells.[155] M-o L-A^+ cells have elevated copy number if they carry either *nuc1Δ* or *ski3Δ* mutations and much higher levels still in a *nuc1Δ ski3Δ* strain. These double mutants with L-A are sick at any temperature and do not grow at all at 37° with ethanol as a carbon source.[155] The similarity of Nuc1 to the mitochondrial EndoG of mammals, which is involved in generating the DNA ladders typical of apoptosis in mammalian cells, leads the authors to suggest that the apoptosis process may have evolved from an antiviral role of Nuc1/EndoG.[155] In the process of meiosis and sporulation, the mother cell (but not the spores) undergoes a programmed cell death apoptosis.[156]

Posttranslational Modification

KEX1 and KEX2 proteases and prohormone proteases

The *kex1* and *kex2* mutants (*KEX*, from killer expression) do not produce the "killer" toxin but do propagate the M_1 satellite dsRNA. The *kex2* mutants also have a defect in mating specific to cells of the α mating type and do not make α pheromone, and *kex2/kex2* homozygous diploids are defective for sporulation.[241] *KEX2* encodes a protease cleaving C-terminal to pairs of basic amino acid residues,[200] and *KEX1* encodes a carboxypeptidase that removes the pair of basic amino acids.[77] These are the cleavages needed to convert the toxin and α pheromone proproteins to their mature forms (Fig. 20.3B). The same specificities are needed to process preproinsulin (Fig. 20.3B), pre-proopiomelanocortin, and other mammalian prohormones, and examination of enzymes with homology to Kex2p led to the discovery of the prohormone processing enzymes (reviewed in Ref.[324]). Kex2p-homologous enzymes are also involved in the proteolytic processing of some mammalian viral proteins.

N-acetylation of Gag by Mak3p—necessary for assembly

MAK3, *MAK10*, and *MAK31* are necessary for the propagation of the L-A dsRNA virus. Mak3p is homologous to *N*-acetyltransferases,[380] and the N-acetylation of the L-A Gag protein is lost in a *mak3* mutant host, resulting in failure of viral assembly.[381] Mak3p recognizes the N-terminal four amino acid residues of Gag.[379] Like L-A, the major coat proteins of Rous sarcoma virus, tobacco mosaic virus, turnip yellow mosaic virus, alfalfa mosaic virus, and potato X virus are N-terminally acetylated. The only three host proteins known to be necessary for L-A propagation, Mak3p, Mak10p, and Mak31p, form a complex,[318] indicating that Mak10p and Mak31p are also involved in N-acetylation of the coat protein. In addition to its action on Gag, the Mak3p acetylase also is responsible for modification of the α5 and α6 proteasome subunits,[215] and both *mak3* and *mak10* mutants grow poorly on ethanol or glycerol.[240]

Viral Assembly

Residues 67-213 of the Pol domain of the Gag-Pol fusion protein binds to the packaging site of a single (+) strand,[147] and the Gag domain of the fusion protein associates with the free Gag protein[149,315] (Figs. 20.3 and 20.4). This leads to encapsidation of a single positive strand per particle if there is only one Gag-Pol fusion protein (or one dimer) per particle.

The *packaging site* recognized is an internal stem-loop sequence about 400 nucleotides from the 3′ end of (+) strands with an A residue bulging from the 5′ side of the stem[131,145] (Fig. 20.3A). Binding requires the stem structure but not the sequence of the stem. In contrast, the sequence of the loop is important. The protruding A residue must be present and must be an A.[145] A similar packaging site, similarly located on the M_1 positive strand, was found[145] by examining the predicted folded structure of the M_1 sequence,[165] and another was found by studying sequences involved in exclusion of M_1.[350] Either the L-A or M_1 stem loops are sufficient for binding; however, the addition of 10 bp from the 5′ side of either one improves the binding and replication substantially.[131,145,350] The added 10 bp gives the L-A and M_1 sites 11 and 10 bp direct repeat sequences, which may be responsible for the improved binding and replication. A site with the same structure has also been found in a similar location in the M_{28} satellite dsRNA.[342]

The L-A and M_1 sites can each serve as a portable packaging signal *in vivo*, directing packaging of heterologous transcripts by L-A virus or by RNA produced from the L-A complementary DNA (cDNA) clone.[145,147] The heterologous transcripts were packaged alone in viral particles, confirming the prediction of the headful replication model that a single positive strand is initially packaged per particle.

Replication (Negative-Strand Synthesis)

Newly assembled viral particles contain an L-A positive strand and are capable of converting it to the dsRNA form when supplied with nucleoside triphosphates.[146] The particles formed in this reaction have all the properties of mature L-A particles and can carry out the transcription reaction.

The opened empty particles (see earlier discussion), when supplied with viral positive strands, Mg^{2+}, nucleoside triphosphates, and a low concentration of polyethylene glycol, carry out negative-strand synthesis to form dsRNA.[150] Only L-A, M, or X positive-strand templates are active in this reaction, supporting the notion that it accurately reflects the *in vivo* reaction.

A maximally active template requires both sequences at the 3′ end of the L-A (or X) positive strand and internal sequences (the internal replication enhancer) overlapping the packaging signal.[136] The 3′ end of L-A positive strands has a stem loop whose structure is necessary for template activity. Whereas the sequence of the loop and that of the 3′ terminal 4 nucleotides are important, that of the stem is not. Despite the requirements for these structures and sequences in the context of L-A, the 3′ terminal 33 nucleotides of M_1's positive strand can substitute for the L-A 3′ end, although there is little or no similarity between the two sequences[136] (see Fig. 20.3). The internal replication enhancer and the 3′ end site must be bound together for optimal template activity in the replication reaction.[152] However, they need not be covalently attached and can simply be hydrogen bonded. This suggests that the RNA polymerase binds first to the internal site and is thus brought close to the 3′ end site where polymerization is to begin.

Because the RNA-dependent RNA polymerase consensus domains defined by Kamer and Argos[205] have since been found in essentially all positive ssRNA and dsRNA viruses examined, the detailed mutagenesis of the most highly conserved of these regions was carried out using the L-A cDNA clone.[316] This has defined the extent of the domains necessary for the propagation of the M_1 satellite dsRNA. Interestingly, homologous regions from reovirus or Sindbis virus RNA polymerases could partially substitute for that of L-A.[316]

Partitiviridae

The *Partitiviridae* have a bipartite genome with the two segments separately encapsidated in particles containing the proteins encoded by both segments.[390] This group includes viruses of the filamentous fungi *Penicillium* and *Aspergillus* and the mushroom *Agaricus*, as well as many other fungi. It also includes a large group of plant cryptoviruses,[32,287] whose genome is dsRNA and whose biology is much like that of the systems discussed in detail here.

Strain 2H of *Atkinsonella hypoxylon* has three dsRNA segments of 2180, 2135, and 1790 nucleotides. Segment 1 encodes an RNA-dependent RNA polymerase, and segment 2 encodes a 74-kd protein that may be the major coat protein.[291] The third segment does not appear to encode a protein.

Reovirus of *Cryphonectria*

Reoviruses associated with hypovirulence have been found in two strains of *Cryphonectria parasitica* and one strain each of *Rosellinia necatrix* and *Sclerotinia sclerotiorum*.[124,247,293,298] The *C parasitica* mycoreoviruses C-18 and 9B-2-1 have 11 dsRNA segments each ranging in size from about 0.7 to about 4.1 kb and associated with 80-nm virus particles, unlike the vesicles in which the potyvirus-like *Hypoviridae* are found. These 11 segments are present in equimolar amounts, and transmission studies show that either all or none of the segments is transmitted, suggesting that they are parts of a single viral genome. The C-18 and 9B-2-1 genomes are not closely related, and sequencing shows that they are related to the *Coltivirus* group (eg, Colorado tick fever virus).[372] The reovirus particles can be introduced into *Cryphonectria* spheroplasts and transmit the hypovirulence traits.[185] This system adds new dimensions to the study of both hypovirulence and the *Reoviridae*.

A Mycoreovirus Facilitates Infection by Preventing Heterokaryon Incompatibility

One important limitation to the use of mycoviruses to control fungal diseases of plants is the heterokaryon incompatibility systems that each filamentous fungus uses to prevent vegetative asexual matings between all but very closely related strains (reviewed in Ref.[168]). Cellular processes of two fungal colonies (hyphae) will fuse as the colonies grow toward each other and test identity of alleles at 6-12 loci (called het for heterokaryon or vic for vegetative incompatibility). Allele differences at any one of these loci results in programmed cell death of the fused

hyphae and formation of a barrier to further fusions. This system serves as a defense against pathogenic plasmids and viruses (such as the hypovirulence viruses) by limiting cell-cell fusions to strains likely to already carry the same viruses (if any).

Wu et al. reported a reovirus (SsMYRV4, 12 dsRNA segments) of the plant pathogen *Sclerotinia sclerotiorum*, which not only has a hypovirulence effect on the fungal host toward rapeseed plants but also blocks heterokaryon incompatibility that would otherwise limit its spread.[415] Heterokaryon incompatibility requires the induction and action of certain G proteins,[252] most of whose induction is blocked by the virus.[415] The virus also lowers the expression of many of the genes containing HET domains.

Single-Stranded RNA Viruses

Traditionally, viruses are classified as ssRNA viruses if the encapsidated form is ssRNA. However, the CHV-1 hypovirus RNA are found in vesicles,[109,177] *Mitoviridae* are in mitochondria[184] not encapsidated, and 20S RNA, 23S RNA are *Narnaviridae*, naked RNA viruses, or nearly so.[159,406] In addition, for the many viruses discovered by sequencing projects, virus particle information is not yet available. As a result, viruses with homology to ssRNA viruses are so designated in such studies and here.

Viruses Reducing Virulence of Chestnut Blight Fungus

The American chestnut tree was devastated by the accidental introduction, in 1905, of the pathogenic fungus *C parasitica*, along with an oriental variety of the chestnut tree (reviewed in Refs.[186,290]). The fungus virtually eliminated the upper parts of the chestnut trees in the eastern United States, although the root systems were not killed and shoots continued to emerge, only to have their growth limited by the reattack of the fungus. *C parasitica* had a similar effect in Europe; however, in the 1950s, the emergence of fungal strains in Italy with markedly reduced virulence to the trees was noted. These "hypovirulent" strains (Fig. 20.5), later also found in Michigan, could transmit their hypovirulence by hyphal anastomosis to virulent strains, and this was used successfully for the biologic control of chestnut blight in Europe. Hypovirulent strains also show decreased asexual spore formation, decreased production of laccase (a phenol oxidase possibly involved in pathogenesis), and reduction in pigment formation (reviewed in Ref.[290]).

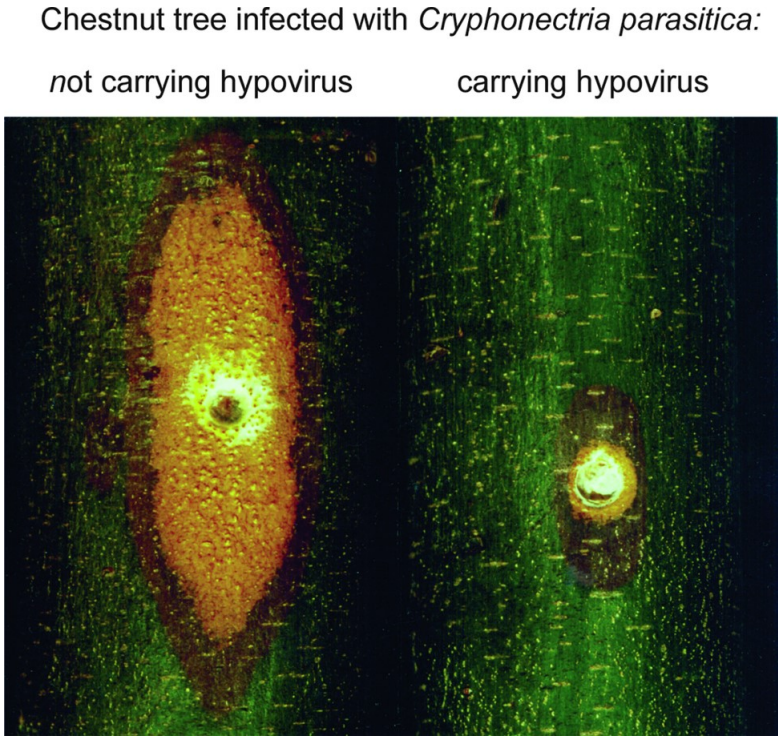

FIGURE 20.5 **Virulent (uninfected, left) and hypovirulent (virus-infected, right) *Cryphonectria parasitica* (chestnut blight fungus) inoculated into chestnut trees**. Infection of the fungus by any of several hypoviruses attenuates its virulence toward the trees. (Courtesy of Dr. Donald Nuss.)

The cytoplasmically inherited factor that reduced the pathogenicity of the fungus and produced these hypovirulence-associated traits was CHV1-EP713, a single segment dsRNA related to the (+) ssRNA plant potyviruses and thus called an ssRNA virus[8,95,224,275] (reviewed in Ref.[290]). CHV1 is the type species of the genus Hypovirus, and four other related Hypoviruses have been found in *C parasitica* as well as many related isolates from other fungi. Not all Hypoviruses reduce host virulence, but many do. Other viruses with a similar hypovirulence effect include the reoviruses.

Genome Structure

CHV1-EP713 is a 12,712-bp molecule whose positive strand has two long ORFs: ORFA and ORFB[347] (Fig. 20.6). Each ORF encodes a papainlike cysteine protease in its N-terminal portion that self-cleaves the primary translation products at least once[69,348] (Fig. 20.6). ORFA and ORFB overlap by a single nucleotide, with the UAA termination codon of the first overlapping with the AUG codon of the second,[347] resulting in a reinitiation mechanism for the expression of ORFB.[174] The translation of ORFA is initiated by internal ribosome entry.[64]

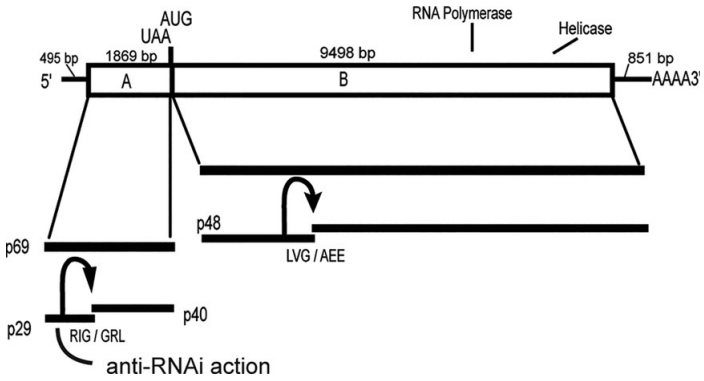

FIGURE 20.6 **Coding information and protein processing of the *Cryphonectria parasitica* virus, L**. The sites of action of the p29 and p48 proteases are indicated. This virus is now designated CHV1-EP713, the type member of the *Hypoviridae*. (Adapted by permission from Nature: Nuss DL. Hypovirulence: mycoviruses at the fungal-plant interface. *Nat Rev Microbiol*. 2005;3(8):632-642. Copyright © 2005 Springer Nature.)

Virus Replication in Intracellular Vesicles

Unlike other mycoviruses, CHV1 is not associated with virus particles but is found in intracellular vesicles,[109,177] with an RNA polymerase activity producing both ssRNA and dsRNA.[137] The vesicles have markers typical of the *trans*-Golgi network.[197]

RNAi in *Cryphonectria parasitica*, *Aspergillus nidulans*, *Sclerotinia sclerotiorum*, *Neurospora crassa*, and *Saccharomyces cerevisiae*

The chestnut blight fungus has an RNA interference (RNAi)-based antiviral system that limits the effects of infection by the hypovirus CHV1-EP713.[345,369,427] *Cryphonectria parasitica* has two homologs (*dcl1* and *dcl2*) of Dicer, the dsRNA-specific endoribonuclease that produces 21- to 24-bp fragments from larger dsRNAs (viral replicative forms or hairpin molecules), and four homologs (*agl1* to *agl4*) of Argonaute, the protein that brings these dsRNA fragments to the complex that "slices" or (at least in animals and plants) blocks translation of target RNAs. The *Cryphonectria* antiviral system involves only *dcl2* and *agl2*. Hypovirus infection induces transcription of both *agl2* and *dcl2*, with the induction of *dcl2* transcription dependent on *agl2*.[369] This antiviral response is muted by the viral p29 protein,[290,344,369] a papainlike protease homologous to the potyvirus suppressor of RNA silencing.[206] Mutation of *dcl2* or *agl2* also prevents accumulation of defective interfering viral particles and blocks viral recombination.[369,426]

In *Aspergillus nidulans*, *Sclerotinia sclerotiorum*, and *Neurospora crassa* RNAi systems have also been defined.[176,187,278] These systems act to limit virus replication. *S cerevisiae* lacks the RNAi system, but the closely related *Saccharomyces castellii* has it.[112] Expressing Dicer and Argonaut from *S castellii* in killer *S cerevisiae* results in loss of M dsRNA and L-A (but not L-BC). The presence of RNAi systems in yeast species correlates with the absence of killer systems, leading the authors to propose that the advantage of having a killer system resulted in loss of the RNAi system.[112] An alternative explanation suggested for the *Kluyveromyces lactis* DNA plasmid-based killer system[274] is that the toxin is there to punish the occasional cell in a clone that loses the plasmid/virus. In this model, the killer systems fail to establish infection in species with RNAi.[400] Wild *S cerevisiae* are only rarely killers or resistant to killing[285] and the killing ability of cells requires a large majority of killers over nonkiller to be effective, both facts arguing against the first model.

Virus Induction of Hypovirulence

CHV1-EP713 infection produces an array of phenotypes, not all of which are responsible for hypovirulence. For example, expression of laccase, a phenol oxidase believed to be involved in pathogenesis, is repressed by CHV1-EP713 infection[66,319] via the p29 protease,[68] but this effect is neither necessary nor sufficient to cause hypovirulence.[84]

G protein signaling is central in the induction of hypovirulence by CHV1-EP713. Hypovirus infection leads to decreased expression of the host Giα (an inhibitory subunit of G protein) and a concomitant increase in cAMP levels.[54,65] Moreover, disruption of *cpg-1*, the gene for Giα, results (in an uninfected strain) in elevated cAMP levels, total loss of virulence, and the development of the same associated phenotypes seen on virus-induced hypovirulence.[157]

Infectious Complementary DNA Clones and Biological Control of Chestnut Blight

Introduction of complete cDNA clones of the CHV1-EP713 genome under control of the *C parasitica* glyceraldehyde-3-phosphate dehydrogenase promoter resulted in both a complete hypovirulence phenotype of the fungus and the launching of the RNA replicon in a form transmissible to other strains.[67] In addition to its usefulness in studying the mechanism of the effects of CHV1-EP713 dsRNA on the cell, this infectious cDNA clone method is an important advance in attempts to control chestnut blight. Although artificial inoculation of trees with hypovirulent fungal strains in Europe resulted in spread of the hypovirulence dsRNAs to virulent strains and control of the blight, this approach has not succeeded in the United States. CHV RNA spreads by fusion between fungal cells, a process called *hyphal anastomosis*, but not by sexual crosses.[7] As discussed above, hyphal anastomosis (heterokaryon formation) is limited by vegetative (heterokaryon) incompatibility determined by alleles at several different *vic* loci. The number of *Cryphonectria parasitica* compatibility groups is apparently much greater in the United States than in Europe,[277] limiting the spread of the hypovirulence virus. In fact, limiting the spread of debilitating viruses is the presumed function of this heterokaryon incompatibility system (see section [Het-s]—a Prion of *Podospora* Responsible for a Normal Function).

In contrast, infectious cDNA incorporated into the *C parasitica* genome, while generating dsRNA replicons, will also naturally spread to other

mating and vegetative compatibility groups through sexual transmission.[67,290] It is thus expected to be more effective in the biological control of chestnut blight than the natural virus (reviewed in Ref.[290]). Another approach is to make strains missing most of their *vic* loci, and therefore able to form heterokaryons with almost any strain.[367,424]

The virus launching from a cDNA approach has also been used to introduce CHV1-EP713 into other plant-pathogenic fungi of the order *Diaporthales*.[337] Infection produced both phenotypic changes and a decrease of virulence in these heterologous hosts. There are, of course, a wide variety of plant-pathogenic fungi, and many cases of hypovirulence due to infecting viruses have been found (reviewed in Ref.[183]).

Negative Strand RNA Mycoviruses

A search of sequence databases revealed chromosomal sequences from *Erysiphe pisi* (pea powdery mildew fungus) homologous to the L-protein (RNA-dependent RNA polymerase) of the Mononegavirus group (includes the (–) ssRNA single segment viruses rabies, measles, mumps, Ebola, and respiratory syncytial viruses).[222] Also, transcriptome shotgun assembly sequences of *Sclerotinia homoeocarpa* (an ascomycete fungus causing dollar spot of grasses) revealed long contigs similar to the L-protein and methyltransferase (capping enzyme) of Nyamanini virus, a Mononegavirus of birds.[222] These sequences were not found in two sequence libraries of *Sclerotinia homoeocarpa*, suggesting that they were from a virus, not the genome.[222]

Such a Mononegavirus was then found in several hypovirulent isolates of *Sclerotinia sclerotiorum* (a plant pathogen), and transfection of virulent *Sclerotinia sclerotiorum* strains made them hypovirulent.[248] The filamentous viral morphology and sequence characteristics of this *Sclerotinia sclerotiorum* negative-stranded RNA virus 1 (SsNSRV-1) were typical of the Mononegaviruses.[248] Transcripts are made of each of the six open reading frames. Similar viruses were found in the plant pathogens *Fusarium graminearum*[394] and *Botrytis cinerea*.[179]

Narnaviridae 20S RNA, 23S RNA, and Mitoviruses

20S RNA and 23S RNA (and their dsRNA forms W and T) are low copy ssRNAs in normal cells but massively amplified in sporulation medium (acetate, no nitrogen source) or at 37 °C.[203,396] Each shows nonchromosomal inheritance and is amplified independent of sporulation/meiosis competence of the host cell.[164,396] 20S RNA[267] and 23S RNA[130] are independent RNA replicons whose copy number is inducible. The sequences of 20S (2.5 kb)[268,326,327] and 23S RNAs (2.9 kb)[129,326] shows that they encode related RNA-dependent RNA polymerases with some similarities to the RNA-dependent RNA polymerases of RNA phages and RNA viruses.

20S RNA is linear,[325] is cytoplasmic, and lacks a coat protein[406] but is found in a 1:1 complex with its encoded 91-kd RNA polymerase.[158] These complexes synthesize 20S RNA and its complementary strand.[159] An infectious clone of 20S RNA has now been developed and used to show the importance of terminal nucleotides.[132] The RNA polymerase binds to three sites on 20S RNA, at the 5′ end, the 3′ end, and in the middle of the sequence, and all three sites are important for propagation of the genome.[141] Intermediates in replication are positive and negative single strands, not dsRNA.[148]

23S RNA is likewise associated with its encoded RNA polymerase in extracts,[128] and an infectious cDNA clone of 23S RNA has been developed and used to study the terminal sequence requirements for replication.[130]

The copy numbers of 20S RNA and 23S RNA are controlled by media conditions, requiring acetate as the carbon source and the absence of a nitrogen source for their 10 000-fold induction.[128,203,267] Both are also induced by growing cells at 37 °C, and at least 20S is repressed by the *SKI* system.[267,396] The *SKI* effect apparently reflects the absence of 3′ poly(A) on 20S mRNA (see earlier discussion).

Cryphonectria parasitica Mitochondrial RNA Replicon NB631

Polashock and Hillman[305] have described a mitochondrial dsRNA species in *C parasitica* strain NB631 (*Mitoviridae*). Assuming the mitochondrial genetic code (UGA = Trp), the NB631 dsRNA has a single long ORF that encodes an RNA-dependent RNA polymerase. This RNA polymerase is most closely related to those of 20S and 23S RNAs, and all are more closely related to RNA phage such as Qβ, than to RNA viruses of higher organisms.[304,327] The degree of similarity is such that these elements must be very close relatives. Nevertheless, 20S RNA and 23S RNA are primarily ssRNA replicons, whereas NB631 has been identified as a dsRNA element. NB631 dsRNA is transferred by hyphal anastomosis, like other *C parasitica* RNA replicons; however, unlike those, and other viruses of filamentous fungi, it is also efficiently transmitted by meiotic spore formation if the female parent had the virus.[304]

Hadaka [naked] Viruses and *Yadokari [home-borrowing] Viruses*

Like the *Narnaviruses*, the *Hadakaviruses* lack a coat, but have 10 or 11 viral segments of (+) ssRNA, not closely related to the single segmented *Narnaviruses*.[338] Members of this group have been found in *Fusarium oxysporum* and *Fusarium nygamai*. In spite of lacking a capsid, the virus segments stably propagate together.

In a phytopathogenic fungus, *R necatrix*, a Calicivirus-related (+) ssRNA single-segment virus has been found encoding its own RdRp, not encoding a coat protein, but instead is encapsidated in the coat of a totivirus in the same strain.[425] This is distinct form the usual helper virus-satellite relation in which the satellite may encode nothing (eg, defective interfering particles) or proteins not involved in its replication (eg, the killer M satellite dsRNAs). In this case, the *Yadokari* [home-borrowing] virus depends on the *Yadonushi* [home-owning] virus for its coat, but incorporates its own RdRp in the viral particles.[425]

A number of other interactions of mycoviruses with their hosts and with each other are being described, although in most cases, the mechanisms involved are as yet unclear, all ably reviewed in Ref.[183].

Chromosomal Genes Affecting Mycoviruses

Infection of Fusarium graminearum with the 6.6 kb ssRNA hypovirulence virus FgV1 leads to overexpression of the HEX1 protein, the major component of the Woronin body, a peroxisome-derived structure that acts as a channel between cells controlling cytoplasmic transfer.[359] In the absence of the virus, deletion of HEX1 does not affect growth but decreases the virulence of the fungus for wheat. However, hex1Δ strains carrying FgV1 have lower levels of viral RNA and less prominent hypovirulence.[359] The HEX1 protein binds specifically to the 5′ and 3′ ends of the viral (+) strand, suggesting that the effect on viral propagation is direct.[358]

Single-Stranded DNA Gemini-like Mycovirus

Sclerotinia sclerotiorum is a pathogen of many crop plants, and among hypovirulent isolates one was found to harbor a single-stranded circular DNA virus which was shown to be the basis for the hypovirulence.[421] Like the plant-pathogenic Geminiviruses, this *Sclerotinia sclerotiorum* hypovirulence-associated DNA virus (SsHADV-1) encodes proteins on both strands, the capsid protein on the (+) strand and a replication initiation protein (Rep) on the (−) strand. SsHADV-1 Rep is most homologous to those of three viruses in the genus *Mastrevirus* in the family *Geminiviridae*. The SsHADV-1 particles are 20-22 nm in diameter without the twinned appearance that gives the *Geminiviridae* its name.[421] This is the first DNA virus to be found in fungi.

SsHADV-1 is exceptional among mycoviruses in having an extracellular phase and can pass during co-culture between strains of *Sclerotinia sclerotiorum* that cannot fuse cellular processes.[421,422] Like the plant-infecting *Geminiviridae*, SsHADV-1 can also infect an insect, the damselfly *Lycoriella ingenua* that feeds on fungi, from a virus-infected fungal strain.[249] The virus replicates in all stages of the insect life cycle (or insect tissue culture cells), and can transmit to an uninfected fungal host. Thus, the virus uses the insect as a vector, like the plant-pathogenic *Geminiviridae*.[249]

Retroviruses (Retroelements)

Retroviruses, Retrotransposons, Retroposons, and Retrointrons

Retroelements (Table 20.1) all share their use of reverse transcriptase in their propagation. The retrotransposons of fungi and parasitic microorganisms resemble mammalian retroviruses in all essentials except for their lacking an *env* (envelope) gene and, in part for this reason, are restricted to propagation without leaving the intracellular environment (Fig. 20.7). Nonetheless, the frequency with which these cells mate in nature is high enough that most of these elements are widely distributed in their respective species. The retroposons are one step further removed in that they lack the long terminal repeat (LTR) structure, and their transposition process differs from those of the other groups. These elements resemble the mammalian LINE elements but have the advantage of readily detectable transposition and facile host genetics for their study. For example, Tad, of *Neurospora*, can retrotranspose between nuclei of a heterokaryon.[218] Excellent reviews of retrotransposons are available.[34,85,162,393]

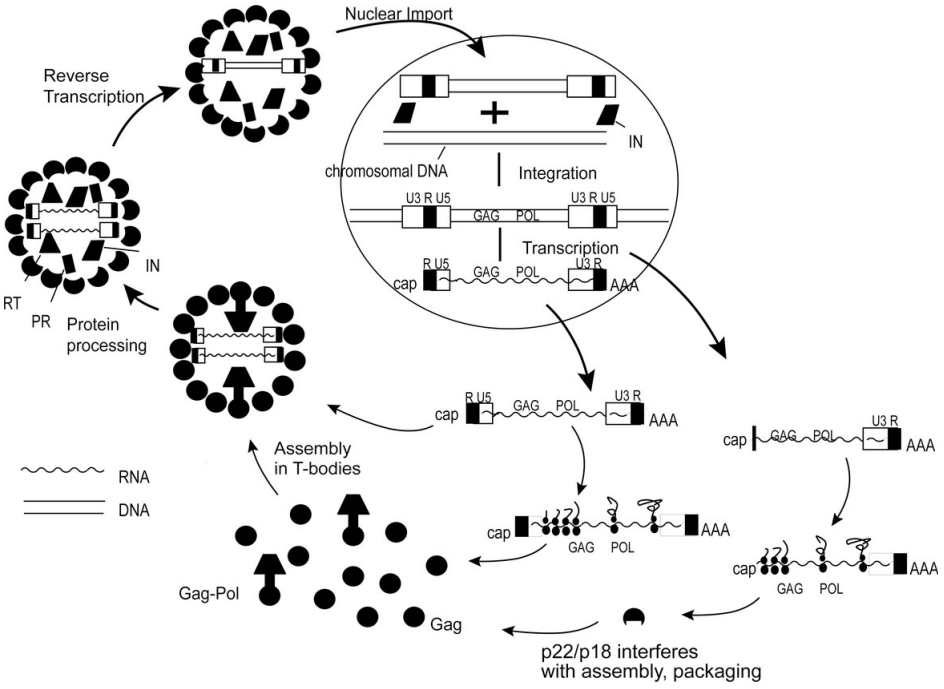

FIGURE 20.7 **The Ty replication cycle**. It is likely that all retrotransposons follow this cycle.

A group of reverse transcriptase encoding introns in mitochondrial DNA of *S cerevisiae* has also been identified[210] (reviewed in Refs.[236,237]). These are introns aI1 and aI2 of the cytochrome oxidase subunit I (COX1) gene. These introns are capable of retrotransposition both into their normal location and into heterologous locations, both *in vitro*[210] and *in vivo*.[281] These retroelements lack LTRs and have only a single ORF. Their properties suggest that introns may have begun as parasitic elements.

The retrotransposons are also divided based on amino acid sequence homologies and gene order in the *pol* domain into the *copia*-like and the

gypsy-like elements. *Copia* and *gypsy* are retroelements of *Drosophila*. Because Ty3 is more similar to gypsy than to Ty1, it is likely that Ty3 and Ty1 entered the yeast genomes at different times and that horizontal transfer of these elements occurs.

Structure of Tys

The Ty elements of *S cerevisiae* each have LTRs of 245-371 bp, separated by a unique region of 4.7-5.6 kb[33] (Fig. 20.8). The major Ty RNA transcript begins within the 5′ LTR (at base 241 from the 5′ end in Ty1) and ends at base 289 of the 3′ LTR, 45 bp from the 3′ end of the element (see Fig. 20.8). This provides the basis for the conventional division of retroviral LTRs into the U3 region (present only at the 3′ end of the Ty RNA but located at the 5′ end of the LTR), the R region (repeated at both ends of Ty RNA), and the U5 region (present only near the 5′ end of Ty RNA but at the 3′ end of the LTR).

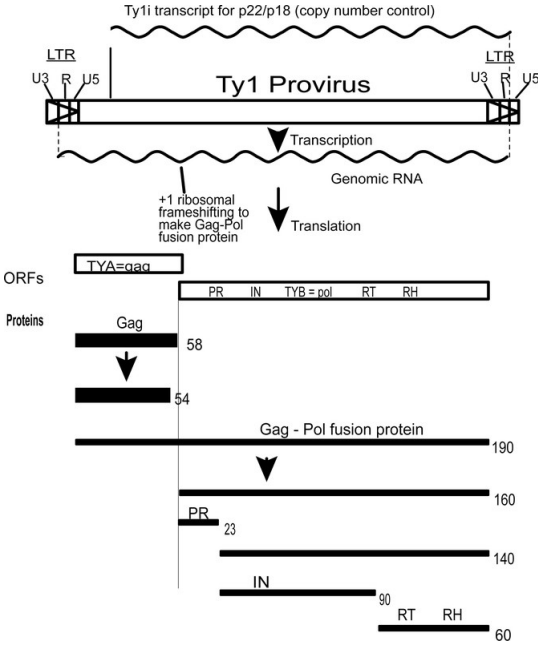

FIGURE 20.8 **Genome structure and expression of Ty1**. Gag and Gag-Pol are the primary translation products, which are processed by cleavage with the viral protease to form the proteins shown. (Adapted with permission of American Society for Microbiology from Garfinkel DJ, Hedge AM, Youngren SD, et al. Proteolytic processing of *pol-TYB* proteins from the yeast retrotransposon Ty1. *J Virol*. 1991;65(9):4573-4581; permission conveyed through Copyright Clearance Center, Inc.)

Ty1 through Ty4 all have two overlapping ORFs: TYA, corresponding to *gag*, and TYB, homologous to the *pol* of mammalian retroviruses. Like mammalian retroviruses and *gypsy*, Ty3 has the gene order protease (PR)–reverse transcriptase (RT)–RNase H (RH)–integrase (IN) in the *pol* ORF,[178] whereas Ty1 and Ty2 have the *copia* order PR-IN-RT-RH.[71]

Replication Cycle of *Saccharomyces cerevisiae* Ty Elements

The Ty replication cycle resembles that of mammalian retroviruses but begins and ends with the integrated form of the genome (see Fig. 20.7). Ty transcripts made with RNA polymerase II are translated to make the Gag and Gag-Pol fusion proteins. These proteins assemble, packaging the Ty RNA to make particles that are homologs of the core particles of retroviruses. The reverse transcriptase and RNase H make a dsDNA copy of the genome, and integrase inserts this into the genome, producing a short repeat of the chromosomal integration site.

Reverse Transcription

The reverse transcriptase and RNase H–homologous domains are present in the Ty1 particles as a 60-kd protein that is produced by proteolytic processing of the 190-kd Gag-Pol fusion protein[160] (see Fig. 20.8). Isolated Ty1 particles have reverse transcriptase that is active on either the endogenous Ty1 RNA or externally added templates. The Ty particles are also open to externally added enzymes, indicating that they are not impermeable shells but have pores. This porous structure may help explain how pseudogene formation can occur.[101] Extraction of active polymerase requires that the C-terminal part of integrase be covalently attached, reflecting the likely cooperation of reverse transcriptase and integrase in virions.[408]

The mechanism of reverse transcription of Ty RNA is largely the same as that for mammalian retroviruses. The primer for reverse transcription is $tRNA_i^{Met}$, with ten nucleotides of the 3′ acceptor stem complementary to a site on the Ty1 positive strand just 3′ to the 5′ LTR, called the negative primer binding site (–PBS).[52] This tRNA is specifically packaged in viral particles. Interestingly, although the primer function of the initiator tRNAMet depends on its complementarity with the –PBS, its packaging is independent of this complementarity.[52] This indicates that the $tRNA_i^{Met}$ is recognized by some other component of the Ty virus particle and that it is probably not the acceptor stem of the tRNA that is recognized. Ty1 cDNA synthesis unexpectedly requires *DBR1*, encoding the enzyme that debranches the lariat structure produced by intron excision by cleaving the 2′-5′ linkage at the branchpoint.[51] Ty1 is reported to have a 2′ to >5′ linkage between the ribonucleotide at the 5′ end of Ty1 RNA and an internal Ty1 ribonucleotide, explaining the requirement for Dbr1p action,[57] but the presence of the branched structure has been questioned.[76]

Integration

Ty3 shows a tight specificity for target sites, integrating 16 or 17 bp upstream of the 5′ ends of tRNA coding regions.[49] The DRE element of *Dictyostelium discoideum* and the spliced leader-specific elements SLACS, CZAR, and CRE1 of trypanosomes have a similar integration target specificity.[3,154,259] Other genes transcribed by RNA polymerase III, such as 5S rRNA and U6 small nuclear RNA, similarly are targets for Ty3.[49] The basis for this site specificity is a specific interaction of Ty3 integrase with the PolIII transcription factor TFIIIC[11] and recognition of the TPB and Brf1 subunits of TFIIIB.[420] Thus, the integration apparatus recognizes the transcription apparatus, not the transcription initiation site or the promoter itself.

Other Tys are capable of insertion at many different sites, with Ty1 producing a five-bp duplication of the target DNA[139]; however, these sites tend to be within 750 bases of a tRNA gene or other RNA polymerase III–transcribed gene.[214] Ty1 insertions in RNA *pol* II–transcribed genes tend to be in the 5′ part of the *URA3*, *LYS2*, and *CAN1* genes,[122,245,286] with control regions targeted far more often than the ORFs.

An *in vitro* integration system using Ty1 viral particles produced from an element carrying a copy of the *Escherichia coli supF* gene and, as target, λ DNA from a multiple amber mutant suppressible by *supF* shows that linear dsDNA substrates carrying the terminal 12 bp at each end of the LTR are sufficient for the integration reaction to occur at normal efficiency.[123] There are also no nucleotide requirements. The substrate DNA must have 3′ hydroxyls, suggesting that the reaction involves covalent attachment of these 3′ ends to the target DNA. Unlike mammalian retroviruses, Ty1's integrase does not remove two terminal bases in the process of integration,[123] probably because in Ty1 the primer binding site is immediately adjacent to the U5 part of the LTR. In fact, the purified integrase protein is capable of carrying out an integration model reaction without other components of the viral particle.[279]

Expression of Tys

Control of Transcription

Transcription of Ty1, Ty2, Ty4, and Ty5 is inhibited in diploid cells by the action of the a1/α2 encoded by the *MAT* locus.[73] In the wild, the haploid phase for yeast is transient, and cells soon mate and spend most of their time as diploids. The 20 times higher transcription of Ty1 in haploids may be a preparation for mating and the possible opportunity to hop into a new genome.

Transcription of Ty3 and Ty5 are derepressed by exposure of cells to the mating pheromones.[72,208,391] Ty3 transposition is, in fact, induced in mating cells, and Ty3 transposing from the genome of one mating partner to that of the other occurs at high rates.[219] Here, the interpretation of preparation to transpose into the potentially Ty3-free genome of the mating partner seems clear.

Filamentous growth, associated with starvation, also mobilizes Ty as a result of elevated transcription produced by the Kss1 MAP kinase cascade.[74] Again, a stressful condition results in Ty mobility.

DNA damage, induced by ultraviolet irradiation or 4-nitroquinoline-1-oxide, also induces Ty1 transposition by inducing transcription of the element.[40] Is this effect adaptive for Ty1 as a first step in finding a new home, or is it a consequence of a failure of the host anti-Ty system?

Retroelements all must cajole the cellular RNA polymerase II into transcribing their proviral form to make viral RNA. They are constrained, however, to place the controlling sequences inside the limits of the element. Whereas most mammalian retroviruses have enhancers in their LTRs upstream of the start site of transcription, Ty1 and Ty2 elements have major transcriptional control sequences downstream of both the transcript start site and the translation start site, most of them inside the unique region. Ty1 has two major downstream sites responsible for its haploid-specific transcription: the sterile-responsive element (SRE) binds Ste12p, and another site (block II or PRTF) binds Mcm1p.[73,125]

Effect of Ty Insertion on Cellular Genes

Insertion of Ty elements into the control regions of cellular genes can activate, inactivate, or alter the control of the target gene[126,410] (reviewed in Ref.[242]). The Ty insertions often move the normal regulatory sequences 5 kb away from the target gene, eliminating the normal regulation. Insertions of Ty whose 5′ end is close to the 5′ end of the target gene impose the Ty transcriptional control on the target gene. This produces a divergent transcription of the Ty element and the target gene; however, both are under mating type control because of the effect of the Ty control region.

Chromosomal Genes Regulating Ty Transcription

Mutant cellular genes that have come under control of Ty1 have been used extensively to investigate the cellular genes affecting the transcription of Ty1 itself.[70,413,414] Second-site mutations (suppressors) that restore the normal expression of the target genes have defined a large group of genes, called *SPT*, that include the TATA binding factor TFIID, the genes encoding histones, and many general transcription factors with effects on many genes (eg, *SNF2*, *SNF5*, *SNF6*, *GAL11*, *SIN1*). In addition, the control of the target genes by mating type and mating pheromones have led to studies of effects of the mating type and pheromone control pathway genes on Ty itself. For example, *STE12* controls Ty1 transcription, as do the genes upstream of *STE12* in the mating type control kinase cascade.[127]

+1 Ribosomal Frameshifting

Like mammalian retroviruses, Ty elements direct the synthesis of a Gag protein and a Gag-Pol fusion protein (see Fig. 20.9). For reasons that are not yet clear, each of the *Saccharomyces* Ty elements uses +1 ribosomal frameshifting, whereas animal retroviruses all use −1 frameshifting (or read-through of a terminator) to make Gag-Pol (reviewed in Ref.[366]). As shown by studies of the L-A dsRNA virus, yeast can perform −1 ribosomal frameshifts by the same simultaneous slippage mechanism used by retroviruses.

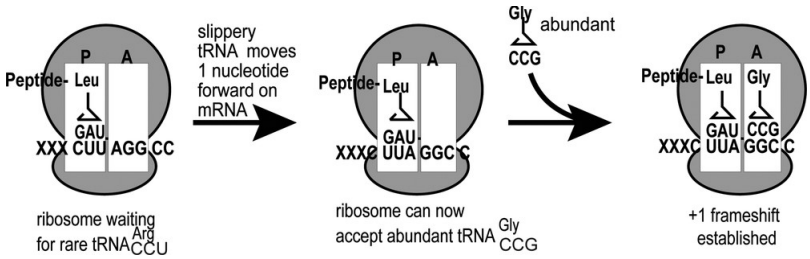

FIGURE 20.9 **Mechanism postulated for +1 ribosomal frameshifting of Ty1**. Ty3 frameshifting involves the new tRNA pairing in the +1 frame without a shift of the peptidyl transfer RNA as for Ty1.[392] (Adapted from Belcourt MF, Farabaugh PJ. Ribosomal frameshifting in the yeast retrotransposon Ty tRNAs induce slippage on a 7 nucleotide minimal site. *Cell*. 1990;62(2):339-352. Copyright © 1990 Elsevier, with permission.)

The mechanisms of the +1 frameshifts in Ty1 or Ty2 (see Fig. 20.9) and Ty3 all involve the combination of starvation for a rare tRNA and an unusual tRNA able to perform the frameshift.[24,140] The Ty1 slippery site on the mRNA is CUU-AGG-C. The low abundance of $tRNA^{Arg}_{CCU}$ recognizing the "hungry" AGG codon at the ribosomal A site slows ribosomes. The $tRNA^{Leu}$, located at the P site while the A site is waiting for the AGG codon to be occupied, has as its anticodon UAG, and it is capable of pairing with either the 0 frame CUU codon or the +1 frame codon UUA. When it slips into the +1 frame, the GGC codon can be easily recognized by an abundant $tRNA^{Gly}$ species, and the ribosomes then continue in the +1 pol reading frame to make Gag-Pol fusion protein.[24]

Oversupply of $tRNA^{Arg}_{CCU}$ lowers frameshift efficiency[24] and transposition frequency[417] as expected. Deletion of the $tRNA^{Arg}_{CCU}$ gene increases frameshifting as expected, but lowers the frequency of transposition[207] indicating that the ratio of *Gag* and *Gag-Pol* is important and that drugs affecting frameshifting efficiency might be useful as antivirals.

The Ty3 frameshift site is GCG-AGU-U, and, like that of Ty1, is based on a hungry codon in the ribosomal A site, namely AGU.[140] AGU is recognized by a low-abundance $tRNA^{Ser}$, thus the ribosome pauses at this point. However, the $tRNA^{Ala}_{CGC}$ that decodes the GCG codon in the P site cannot slip +1 and re-pair. Rather, it is believed that the valine tRNA simply pairs out of frame.[392] A downstream sequence may stimulate Ty3 frameshifting by interacting with rRNA.[366]

Proteolytic Processing and Phosphorylation

Ty1 Gag is expressed as a primary translation product of 49 kd, most of which is processed by the Ty1 protease to form the 45-kd major particle protein. After Pol is synthesized as a 199-kd Gag-Pol fusion protein, it is processed through several intermediates to form the protease, integrase, and reverse transcriptase–RNase H[160] (see Fig. 20.8).

Packaging and Assembly

The Ty1 RNA site determining packaging has been localized to within a 381-nucleotide region between nucleotides 239 and 620.[418] An RNA pseudoknot at the 5′ end is important for retrotransposition,[35,192] but the RNA structure recognized for packaging and the parts of Gag or Gag-Pol proteins that recognize this region have not yet been determined.[173]

Ty5 and Ty1 mRNA and Gag localize in cytoplasmic particles (T-bodies) thought to be involved in viral particle assembly.[23,53,255,256] Assembly of T-bodies requires components of the mRNA-processing bodies referred to as P-bodies (Dhh1p, Lsm1p, Pat1p); however, T-bodies are distinct from P-bodies in the case of Ty1[53,114] but may be identical in the case of Ty3.[23] Mutants in these P-body components are defective for retrotransposition, as well as for cDNA accumulation. Retrotransposition requires not only the P-body structure but also its RNA-degrading enzymatic activities, such as the decapping enzyme *Dcp1/2* and 5′-3′ exoribonuclease *Xrn1p/Ski1p*.[53,114]

Ty Self-imposed Copy Number Control

Wild strains of *S cerevisiae* carry up to about 40 copies of Ty1, most of which are probably transposition competent, but not more.[89] Moreover, transposition in a host carrying only a single Ty1 is over 100-fold more frequent than the usual host (S288C has 32 Ty1s).[161] In another comparison, 20 copies of Ty1 produced a 4800-fold decrease in transposition.[161] This phenomenon is called copy number control (CNC). CNC is due to each Ty1 making a small amount of a C-terminal fragment of Gag called p22 (and its protease-processed form p18) made from an internally promoted transcript called Ty1i starting at nt 1000 of Ty1 with the same 3′ end as Ty1 genomic RNA[334] (Fig. 20.8). Unlike the genomic Ty1 transcript, the Ty1i transcript is independent of Spt3, a subunit of the SAGA transcription regulation complex required for production of the full-length genomic Ty1 RNA (gRNA).[334]

Both p22 and p18 are incorporated into the T-bodies (Ty1 assembly factories) and Ty1 virus-like particles, and interact with Gag to prevent proper assembly and function of the particles.[80,334,385] Because p22/p18 have the C-terminal part of the capsid domain of Gag, they can assemble with Gag by interacting with its C-terminus, but the absence of the N-terminal domain apparently prevents completion of proper assembly and function.[80] The effectiveness of p22/p18 is proportional to its level in the cell, with the highest levels completely shutting off retrotransposition.[334] Although Ty1 tends to insert at relatively safe (for the host) places, Ty1 copy number is inversely correlated with fitness.[409] Thus the CNC makes Ty1 a smart parasite.

Antisense RNA transcribed from the 5′ end of the Gag ORF and incorporated into viral particles was thought to play a role in copy number control and can indeed inhibit transposition,[28,266] but its status is now uncertain because p18/p22 interference with Ty1 assembly and activity now provides a complete explanation of CNC.[334]

Host Limitations on Ty Transposition Efficiency

Studies of the mechanism and control of transposition have often used a plasmid with a GAL1-promoted Ty1 (inducible on galactose) carrying a marker (such as *HIS3*) in the antisense orientation with an artificial intron in the sense orientation) to facilitate detection of transposition.[86] When Ty1 transposition is induced with such a plasmid, although Ty1 RNA is increased only a few fold over that derived from the normal chromosomal Ty1 copies, the frequency of transposition increases about 100-fold, suggesting a block to transposition that is saturable by Ty1 mRNA.[87] Many host genes have been identified whose mutation derepresses Ty1 transposition, analogous to the effect of ski mutations on RNA virus copy number (see earlier discussion).

Mediator is a large complex that connects DNA-binding transcription activators to RNA polymerase II. Deletion of nonessential subunits of Mediator increase or decrease Ty1 transposition frequencies on the order of 100-fold and show only modest effects on Ty1 antisense transcript or Ty1 sense full-length transcripts, generally not correlated even in direction with the effects on transposition.[335] However, there are substantial changes in Ty1i transcript and levels of p22 and p18 that correlate well with the effects on transposition.[335]

Xrn1p is a 5′-3′ exoribonuclease involved in mRNA decay, and *xrn1* mutants have elevated Ty1i transcript, elevated p18, and decreased Ty1 transposition.[334] Spt3 is a component of the SAGA chromatin-remodeling complex and is necessary for Ty1 full-length transcription. However, *spt3* mutants also have elevated Ty1i transcript and increased levels of p22/p18, which must contribute to their decreased Ty1 transposition.[334]

Loc1p, a nucleolar protein involved in ribosome biogenesis and the asymmetric localization of *ASH1* mRNA between mother and daughter cell, inhibits CNC.[1] Like several mutants in genes for ribosomal proteins, *ash1*Δ strains show modest effects on transposition of lone Ty1s, but substantially decreased transposition in strains with the normal complement (~32 copies) of chromosomal Ty1.[1]

Mutation of the cellular *rad6* gene, encoding a ubiquitin-conjugating enzyme, increases the frequency of Ty transposition at either *URA3* or *CAN1* and changes the distribution of insertions.[301] This effect is not caused by altered Ty transcript levels and was seen even when retrotransposition from a GAL1-promoted Ty1 was studied. Rad6p alters chromatin structure by monoubiquitination of histone H2B on Lys123,[323] suggesting that the Rad6p effect on Ty1 transposition is on the nature of the target. Mutation of *CAC3*, encoding a subunit of chromatin assembly factor I, and *HIR3*, a histone transcription regulation gene,[309] also affect both the frequency of integration and the distribution of insertions by alterations of the structure of the target.[191]

Mutation of the *FUS3* MAP kinase results in a more than 20-fold increased Ty1 transposition frequency.[74] Ty1 transcript levels and primary translation products were not affected by *fus3*; however, virus particle–associated processing products of Pol and Ty cDNA were each elevated 10-fold or more in the mutant. The Fus3 kinase is activated by exposure to the mating pheromones via a cascade of kinases, thus explaining the inhibition of Ty1 transposition by exposure to mating pheromones.[419] Fus3 acts by stimulating the invasive growth pathway.[75]

Mutations in more than 20 genes encoding DNA replication and repair components show derepression of Ty1 retrotransposition (reviewed in Ref.[272]). For example, mutants in *SSL2* and *RAD3*, encoding excision-repair DNA helicases, show elevated retrotransposition,[239] as do mutants in *RRM3*, encoding a helicase that promotes replication through DNA-protein complexes.[88] Rad3 represses Ty1 transposition (28×) even in a cell with only a single Ty1,[161] while it shows a 170-fold repression in a cell with many Ty1s.[239] So at least Rad3 is not working solely (if at all) through CNC,[161] but other mutations have not yet been tested.

These mutations do not elevate Ty1 transcripts; however, the level of Ty1 cDNA is elevated.[88,239,370] This group of mutations is known to activate the DNA damage checkpoint pathway or the DNA replication stress pathway, and their derepression of Ty1 transposition is prevented by mutation of elements of the checkpoint pathways, such as *rad53*, *rad9*, *rad24*, *mec1*, and *dun1*.[88] Thus, DNA damage or replication arrest from a host of sources, by activating the checkpoint pathways, turns on Ty1 retrotransposition. Although the mechanism of this effect is not yet clear, the best explanation appears to be that cellular conditions at the arrest point in the cell cycle are particularly favorable for transposition.[272] There is no evidence that this derepression favors either the yeast host or Ty1.

Ty5 integration is concentrated in regions of heterochromatin, unlikely to be detrimental to the host.[430] A nine-residue section of the Ty5 integrase determines association with Sir4p, a heterochromatin protein, and hence the site specificity.[416] However, this interaction and the corresponding targeting to heterochromatin only occur properly when this targeting domain site is phosporylated at S1095 by a host kinase.[91] Under stress conditions, this control system breaks down, the targeting domain is not fully phosphorylated, and integration is random, producing more serious mutations.[91]

Schizosaccharomyces pombe Retroelements

The Tf1 and Tf2 elements of *Schizosaccharomyces pombe* are unusual in that a single ORF encodes both Gag and Pol.[243] From the primary 140-kd translation product, the viral protease cleaves the proteins that form the viral particles.[244] In view of the strict requirement for the ratio of Gag to Gag-Pol in Ty1[207,417] and retroviruses, the assembly process in Tf1 and Tf2 must be significantly different. In log phase cells, the ratio of Gag to integrase in particles is close to 1, whereas in stationary cells, it is quite high.[10] Selective degradation of the non-Gag protein components alters the ratio after particle formation.[10]

The priming mechanism of Tf1 is also unique. Instead of tRNA priming as for other retrotransposons and retroviruses, Tf1 negative-strand DNA synthesis is primed by the first 11 nucleotides at the 5′ end of the viral RNA, which is complementary to the primer binding site.[246] A hairpin is formed, which is nicked to separate the first 11 nucleotides from the 5′ end of the RNA and give it a 3′-OH that can serve as a primer for strong-stop DNA synthesis.

Although the *piwi* RNAi system controls retrotransposons in mammals and many other organisms, two chromatin-modifying protein-based systems have been identified in *S pombe* that silence *Tf2* elements and *Tf2*-derived LTRs and prevent their recombination that could damage the genome.[45,172] The HIRA complex, a nucleosome-assembly apparatus that includes Hip1, Slm9, and Hip3, is important for the assembly of silencing heterochromatin at various sites, including the *Tf2* retrotransposon.[172] Three homologs of human CENP-B—Abp1, Cbh1, and Cbh2—

are involved in a different chromatin silence system that works by binding to *Tf1* and *Tf2* loci (and lone LTRs) and recruiting the histone deacetylases Clr6 and Clr3 to those sites.[45] In addition to the silencing produced by localized histone deactylation, the CENP-B homologs draw together the *Tf* loci into intranuclear bodies, which may facilitate their silencing.[45]

Candida albicans Plasmid Retrotransposon and Line Elements

Most retrotransposons have minute amounts of cDNA present in normal strains; however, that of *Candida albicans* (pCal) produces 50-100 copies per cell of linear dsDNA in a particular strain of *Candida*.[270] pCal is a member of the Ty1/*copia* group found in integrated form in most strains, and the basis for its high free levels is not yet known. pCal is also unique among retrotransposons in that it uses read-through of a stop codon to make its Gag-Pol fusion, as in murine leukemia virus.[270]

C albicans also carries several families of non-LTR retrotransposons (LINEs), homologous to the mammalian L1 LINE elements.[169] One of these elements, Zorro3, has been shown capable of transposition in *S cerevisiae*.[111] Non-LTR retroelements have also been identified in *Cryptococcus neoformans*.[170]

Prions of *Saccharomyces* and *Podospora*

The concept of an infectious protein arose in studies of the transmissible spongiform encephalopathies (TSEs), such as scrapie of sheep, mad cow disease, and Creutzfeldt-Jakob disease of humans (see Chapter 22). Based on genetic evidence (Fig. 20.10), two non-mendelian genetic elements of *S cerevisiae*—[URE3] and [PSI+]—were identified as prion (infectious protein) forms of Ure2p and Sup35p, respectively.[397] This conclusion is supported by extensive further evidence, and both are transmissible amyloidoses, a remarkably close parallel to the mammalian disease. Two further amyloid-based prions—[Het-s] of the filamentous fungus *Podospora anserine*[81] and [*PIN*$^+$] of *S cerevisiae*[98] —were next found and have been studied extensively. An infectious protein consisting of an enzyme whose active form is essential for activation of its own inactive precursor can also be an infectious protein, and two such cases have been described.[212,235,322] A recently described prion, [SMAUG+], consists of a nonamyloid aggregation of Vts1p, a site-specific RNA-binding protein that stimulates mRNA degradation by inducing its deadenylation.[48] [SMAUG+] cells have increased sensitivity to UV irradiation but better growth at limiting dextrose levels.[48] Several other yeast prions have been reported (Table 20.2).

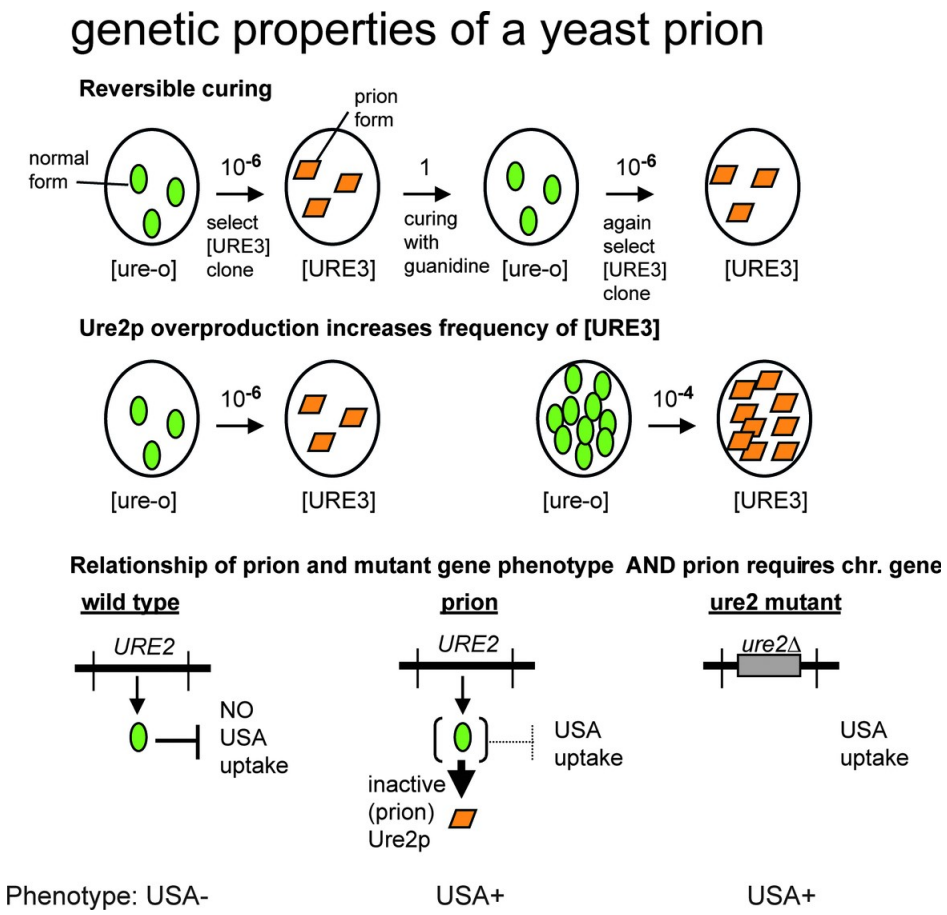

FIGURE 20.10 **Three genetic criteria for a prion**. These are unusual features, expected of a prion but not of a nucleic acid replicon. (Based on Wickner RB. [URE3] as an altered URE2 protein: evidence for a prion analog in Saccharomyces cerevisiae. *Science*. 1994;264:566–569.)

TABLE 20.2 Prions of yeast and fungi

Name	Prion Protein	Normal Function	Prion Phenotype (Variant Dependent)	Reference
[PSI+]	Sup35p	Translation termination, mRNA turnover	Termination codon read-through, slow growth, cell death	397
[URE3]	Ure2p	Nitrogen catabolite repression	Derepressed for transporters and enzyme for poor N sources, very slow growth	397
[PIN+]	Rnq1p	Unknown	Weakly primes [PSI+] generation	98
[SWI+]	Swi1p	Chromatin remodeling	Poor growth on raffinose or glycerol	113
[OCT+]	Cyc8p	Global transcription co-repressor	Derepression of multiple genes	295
[MOT+]	Mot3p	Transcription regulator	Derepression of anaerobic genes	4
[β]	Prb1p	Vacuolar protease	Active protease, survival in stationary phase, sporulation	322
[MOD+]	Mod5p	tRNA isopentenyl transferase	Slow growth, partial resistance to azoles (nocodazole, fluconazole)	371
[SMAUG+]	Vts1p	mRNA degradation	UV-hypersensitive, Improved growth in limiting glucose	48
[Het-s]	HET-s	The prion is the normal form	Heterokaryon incompatibility	81

The [β] prion is the active form of the self-activating enzyme, vacuolar protease B, and is not related to amyloid formation. Vts1p is a nonamyloid aggregate in [SMAUG+] strains. The other prions listed here are all self-propagating amyloids.

Domains from many yeast genes, a plant gene, and a bacterial gene have been shown capable of acting as prion domains replacing the prion domain of Sup35p, a few of which have been shown to be prions (*infectious* proteins) in their native context.[4,47,423]

[URE3]—A Transmissible Amyloidosis of Ure2p

When yeast has a good nitrogen source, such as ammonia, Ure2p, by binding the transcription factor Gln3p, shuts off transcription of genes encoding enzymes and transporters needed for the utilization of poor nitrogen sources, such as *DAL5*, encoding the allantoate transporter.[78] In 1971, Lacroute described a non-mendelian genetic element of *S cerevisiae* called [URE3], which allowed cells to take up ureidosuccinate despite the presence of a rich nitrogen source.[234] Ureidosuccinate is the product of aspartate transcarbamylase (*URA2*) in uracil biosynthesis. Because of chance resemblance to allantoate (a poor nitrogen source), it is taken up from the medium by Dal5p, and *DAL5* is repressed by ammonium via Ure2p action. Thus, [URE3] *ura2* strains can grow on ureidosuccinate in the presence of ammonia, whereas [ure-o] cells (lacking [URE3]) cannot. Fusing the *DAL5* promoter to the *ADE2* ORF allows assaying [URE3] by adenine prototrophy.[340]

Genetic Evidence That [URE3] is a Prion

Three lines of genetic evidence first showed that [URE3] is actually an altered form of Ure2p that has lost its activity in repressing nitrogen metabolic enzymes through Gln3p but has acquired the ability to convert the normal Ure2p to this altered form[397] (see Fig. 20.10). First, [URE3] is efficiently cured by growth of cells on guanidine HCl; however, the curing is reversible in that [URE3] derivatives may again be selected from the cured strain.[397] Second, transient overproduction of Ure2p leads to a 20- to 100-fold increase in the frequency with which [URE3] arises *de novo*.[397] Third, Ure2p is required for propagation of the [URE3] nonchromosomal gene, but the phenotypes of *ure2Δ* and [URE3] are essentially identical.[2,397] These three features are strongly predicted by the prion model, although not if [URE3] were a nucleic acid replicon—a virus or plasmid.[397] These three properties, none of which were known for the mammalian TSEs, also served to identify [PSI+] as a prion form of Sup35p,[397] [PIN+] as a prion of Rnq1p,[98] and other prions.

Ure2p is partially protease resistant in extracts of [URE3] strains, like the amyloid of PrP in brains of TSE-infected animals.[264] Moreover, the generation and propagation of [URE3] was unaffected by the repression or derepression of nitrogen regulation, eliminating some alternative models. [URE3] truly does arise *de novo*, and it is the Ure2 protein, not the *URE2* mRNA or the copy number of the *URE2* gene, that induces [URE3] to arise.[262]

Domains of Ure2p

Ure2p is composed of a C-terminal functional domain, whose structure resembles glutathione-S-transferases (residues 95-354),[37,264,389] and an unstructured N-terminal domain (residues 1-94).[302] Ure2p$^{90\text{-}354}$ is sufficient, if overexpressed, for nitrogen regulation but is rapidly degraded without the N-terminal residues.[351] This stabilization domain is also the part of the molecule responsible for the prion change (the prion domain) and comprises roughly the N-terminal 65 residues.[264] [URE3] is efficiently transmitted by cytoplasmic mixing to cells expressing only Ure2p$^{1\text{-}65}$ from cells expressing the full-length protein. The cells expressing only Ure2p$^{1\text{-}65}$ stably propagate the prion and efficiently transmit it again to cells expressing the full-length Ure2p.[262] On conversion to the infectious amyloid form, the C-terminal domain is largely unchanged in structure,[12] and the prion domain (residues 1-65) assumes an extremely stable amyloid form.[20,377] Residues 66 through 94 remain unstructured in the amyloid form and are called the *tether* because they attach the structured C-termini to the structured amyloid core.

Infectious Amyloid of Ure2p Is [URE3]

Ure2p–green fluorescent protein fusions are aggregated in [URE3] cells, indicating that aggregation is involved in the [URE3] change.[118] The Ure2p prion domain synthetic peptide can form amyloid, and this domain also directs the conversion of the full-length native Ure2p into amyloid[377] (Fig. 20.11). The product of this reaction can seed further amyloid filament formation *in vitro*, indicating that the [URE3] phenomenon is a transmissible amyloidosis of yeast,[377] and filaments of Ure2p can be seen by electron microscopy specifically in cells infected

with [URE3].[365] Ure2p is inactivated for its nitrogen regulation function apparently simply because it is aggregated and thus relatively inaccessible to Gln3p.[19]

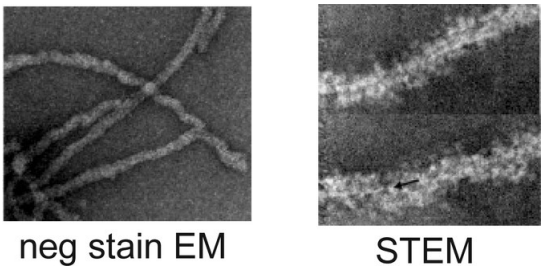

FIGURE 20.11 **Amyloid filaments of recombinant Ure2p**.[20,377] **Left**. Electron micrograph of negatively stained Ure2p amyloid filaments. **Right**. Scanning transmission electron micrograph of Ure2p amyloid filaments[20] with the amyloid core visible (*arrow* in one of the filaments).

Infection by amyloid of recombinant protein was first achieved for [Het-s] and then for [PSI+] (see later discussion). Amyloid formed *in vitro* from recombinant Ure2p efficiently infects yeast cells with [URE3].[38] The infectivity of Ure2p amyloid resides in particles that are no <20 nm in size, a minimum of about 40 molecules.[38] Very large filaments are not easily introduced into yeast cells but contain abundant infectivity that is revealed by sonication. Prion variants of [URE3] were described,[340] and amyloid made from recombinant Ure2p induces the appearance of at least three distinct variants of [URE3].[38] Extracts of each variant are again infectious and induce the appearance of the corresponding [URE3] variant.[38] Fractionation of amyloid of recombinant Ure2p produces some fractions in the 20- to 200-nm range whose infectivity is nearly as high as that of extracts. Infectivity resides entirely in the N-terminal prion domain, again showing its central role.[38]

[PSI+]—A Transmissible Amyloidosis of Sup35p

In 1965, Cox described [PSI+], a non-mendelian genetic element of *S cerevisiae* that increases the efficiency of nonsense suppression by classical tRNA suppressors.[82] Like [URE3] and Ure2p, [PSI+] is reversibly curable,[253,356] transient overexpression of Sup35p increases the frequency with which [PSI+] arises by 100-fold,[58,100] the phenotype of [PSI+] is like that of *sup35* mutants, and *SUP35* is necessary for propagation of [PSI+].[110,378] This logical parallel with [URE3] and Ure2p indicated that [PSI+] is also a prion form of Sup35p.[397]

Infectious Amyloid of Sup35p

Sup35p is aggregated in [PSI+] strains but not in [psi–] strains,[297,299] and the *in vitro* aggregation of native soluble Sup35p seeded by the prion form from [PSI+] cells will continue indefinitely as long as fresh native Sup35p is supplied.[300] The N-terminal 114 residues of Sup35p could propagate [PSI+], and so constitute the prion domain (Sup35N),[378] and as a synthetic peptide spontaneously forms amyloid *in vitro*.[217] In addition, the full-length Sup35p, made in *E coli*, forms β-sheet–rich filaments spontaneously when diluted out of denaturant, a process that can be accelerated specifically by an extract from [PSI+] cells.[166]

Amyloid formed from recombinant Sup35p transmits [PSI+] to cells, inducing the appearance of several prion variants.[216,373] Extracts of cells bearing a variant are infectious and transmit the variant carried by the strain from which the extract was made. Moreover, seeding soluble Sup35p with an extract amplifies amyloid transmitting that prion variant. Adjusting the conditions under which amyloid forms spontaneously strongly biases the variant encoded by the amyloid formed.[373] These results are useful in studies of the structural differences between prion variants.

[PIN+]—A Prion that Generates Other Prions

Derkatch et al. found that whereas overproduction of Sup35p induces the high-frequency appearance of [PSI+] in some strains, others did not show this effect.[99] Their analysis showed that this effect was attributable to the presence of a nonchromosomal genetic element in the strains in which [PSI+] generation was inducible. The Rnq1 (rich in N and Q) protein showed a self-propagating aggregation,[362] and indeed Rnq1 amyloid formation proved to be the basis of the [PIN+] nonchromosomal gene.[98] Overproduction of other N/Q rich proteins likewise has [PIN+]-like activity (without being themselves prions), as does the [URE3] prion.[98] Moreover, [PIN+] also modestly promotes *de novo* generation of [URE3].[39] [PIN+] probably promotes generation of prions by other N/Q rich proteins by rare cross-seeding. Recombinant Rnq1p also forms amyloid *in vitro*,[362] and this amyloid can infect yeast cells with the [PIN+] prion.[296] The promotion of prion seeding by [PIN+] is probably due to cross seeding by the Q/N rich Rnq1 amyloid of the prion domains of the other prion proteins, nearly all of which are also Q/N rich.

[Het-s]—A Prion of *Podospora* Responsible for a Normal Function

Hyphal anastomosis/vegetative fusion/heterokaryon formation (explained above “A mycoreovirus facilitates infection by preventing heterokaryon incompatibility”) depends on allele identity at 6-12 *het* loci (Fig. 20.12) (reviewed in Ref.[339]).

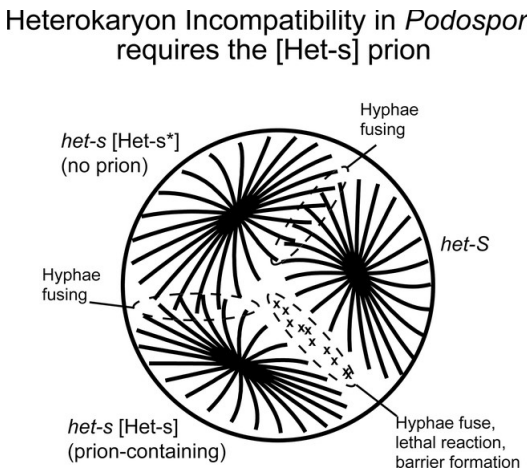

FIGURE 20.12 **The [Het-s] prion of *Podospora anserina* is required for heterokaryon incompatibility**.

(Adapted with permission from Coustou V, Deleu C, Saupe S, et al. The protein product of the *het-s* heterokaryon incompatibility gene of the fungus *Podospora anserina* behaves as a prion analog. *Proc Natl Acad Sci U S A*. 1997;94(18):9773-9778.)

One of the *het* genes of *Podospora anserina* is *het-s*, with alleles *het-s* and *het-S* encoding a 289-residue protein differing at 14 positions,[387] although the difference at residue 33 is sufficient to trigger the incompatibility reaction.[97]

Rizet found that *het-s* strains could show the incompatibility reaction only if they also carried a non-mendelian genetic element referred to as [Het-s].[321] [Het-s] could be "cured" from a male strain by mating; however, from the cured strain, [Het-s] cells arose at a frequency of about 10^{-7}.[22] This is the *reversible curing* criterion for a prion. Coustou et al showed that overproduction of the HET-s protein increased the frequency with which [Het-s] arose, and that the HET-s protein is relatively protease resistant in strains carrying [Het-s]. Moreover, deletion of the *het-s* gene makes a cell unable to propagate the [Het-s] non-mendelian genetic element.[81]

The prion domain of HET-s is the C-terminal residues 218 through 289, of which residues 228 through 289 are unstructured in the native form.[14] The HET-s prion domain is not N/Q rich but forms amyloid filaments *in vitro* that are infectious for cells not carrying the prion.[254] The demonstration that amyloid formed *in vitro* from recombinant HET-s protein was infectious was the first demonstration of its kind for any prion.[254]

Beyond its obvious interest as a new prion, [Het-s] is particularly important because it is the first case in which a prion appears to be responsible for a normal cellular function. Heterokaryon incompatibility is observed in most filamentous fungi, usually controlled by chromosomal genes that have no particularly unusual features. In this case, it requires the [Het-s] prion to be observed. This suggests that other prions may be found responsible for normal cellular functions.

However, another interpretation of [Het-s] is possible, based on a [Het-s]-based meiotic drive system. Mating a female *het-s* [Het-s] strain with a male *het-S* strain results in death of *het-S* meiotic segregants that received [Het-s] cytoplasm.[92] Thus, one could view the [Het-s] prion as a device for the preferential inheritance of the *het-s* allele by a meiotic drive mechanism.

Adjacent to the *het-s/S* gene is *Nwd2*, that includes an N-terminal sequence resembling the HET-s prion domain, and a nucleotide-binding oligomerization domain (NOD)-like receptor sequence. It was suggested that ligand binding by NWD2 induced oligomerization that leads to amyloid formation by the NWD2 N-terminal sequence which, in turn, activates HET-S by seeding its amyloid formation.[94] This hypothesized signaling process was suggested for a large number of fungal and mammalian proteins,[94] and strong support for this idea has now been obtained (reviewed by Refs.[93,317]).

Prion Variants

A single protein sequence can be the basis of many distinct prions, called "strains" in mammals and "variants" in yeast. Mammalian TSE strains were described early on, characterized by different incubation period, symptoms and signs, and distributions of brain lesions.[44] Independent isolates of [PSI+], [URE3], [PIN+], or other prions may likewise have various intensity of phenotype or stability.[39,100,340] These prion variants breed true, and extracts of a strain carrying a given variant of [PSI+] or [URE3] transmit almost exclusively that variant by transformation.[38,216,373] It is clear that different [PSI+] prion variants have different amyloid structures,[374] and one of the main challenges is to determine these structural differences (Fig. 20.13). Variants of [URE3] and [PSI+] have now been found differing in their pathogenicity or sensitivity to excess or deficiency of various chaperones or their sensitivity in normal cells to antiprion systems (see below).

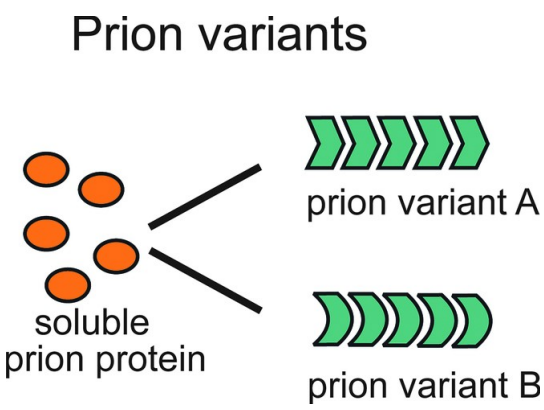

FIGURE 20.13 A single protein sequence can stably propagate any of several prion variants, each corresponding to a different amyloid structure.

It is striking that only one variant of [Het-s] has been described. Because [Het-s] is either carrying out a function for the host or is a part of a meiotic drive system, it is in either case selected in evolution to be a prion. The protein sequence has presumably been selected to efficiently form the single prion variant that carries out this function.

Structures of Yeast Prion Amyloids Explain Variant Propagation

Ure2p and Sup35p prion domain amyloids have the cross–β-sheet structure typical of amyloids, meaning that the β-strands run perpendicular to the filament long axis.[18,221,377] Infectious prion domain amyloids of recombinant Sup35p, Ure2p, and Rnq1p each have a parallel in-register β-sheet structure based on solid-state nuclear magnetic resonance (NMR) studies,[21,352,399] with identical residues in adjacent molecules aligned in a row along the long axis of the filament (Fig. 20.14). The mass per unit length of Ure2p, Sup35p, and Rnq1p prion domain filaments is in each case the one monomer per 4.7 Å expected for this structure.[20,56,102] The diameters of the filaments[217,377,399] show that these filaments must be multiply folded along the filament axis (see Fig. 20.14). This structure is like that found for amyloids of Aβ, amylin, α-synuclein, β2-microglobulin, tau, and most other human pathologic amyloids (reviewed in [258,388]).

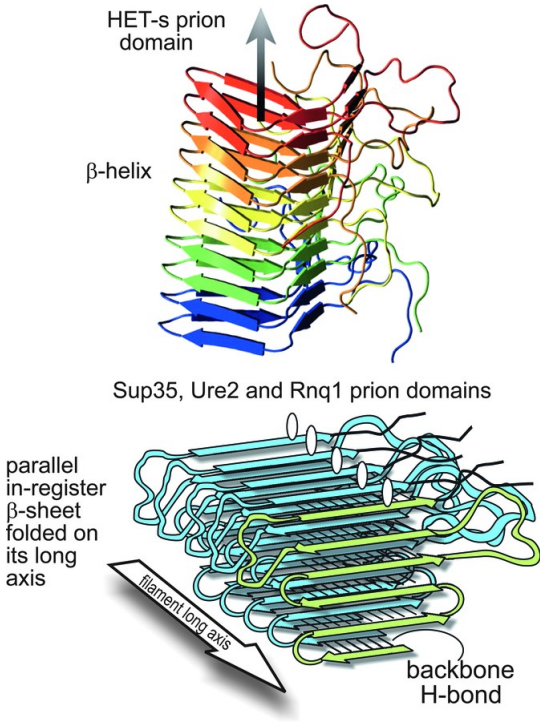

FIGURE 20.14 **Structures of prion amyloids**. **Above:** Infectious amyloid of HET-s$^{218-289}$ has a two-turn β helix structure. (Adapted from Wasmer C, Lange A, Van Melckebeke H, et al. Amyloid fibrils of the HET-s (218-279) prion form a beta solenoid with a triangular hydrophobic core. *Science*. 2008;319(5869):1523-1526. Reprinted with permission from AAAS.) **Below:** Infectious amyloid of Ure2p$^{1-65}$, Sup35p$^{1-253}$, and Rnq1p$^{153-405}$ each have an in-register parallel β-sheet architecture.[21,352,399]

The in-register parallel β-sheet structure is maintained by the positive interactions among identical aligned amino acid side chains—H-bonds between the amide side chains of glutamines or asparagines, H-bonds between the –OH groups of serine or threonine side chains, or hydrophobic interactions among hydrophobic amino acid side chains. These interactions demand that identical residues be aligned. The same interactions direct a monomer joining the end of the amyloid filament to assume the structure of the other molecules in the filament.[404,405] Thus, although any of many different parallel in-register structures may form at the time of filament initiation, the location of the folds of the sheet, and the extent of the β-sheet structure will be propagated as the filament is elongated by this mechanism (Fig. 20.15). This mechanism

[402,404] can explain how a protein can template its own conformation (just as DNA can template its own sequence), and thus how proteins can be genes. Recently, infectious PrP^{Sc} amyloid, the basis of the mammalian prions, has been shown to have an in-register parallel β-sheet structure.[225]

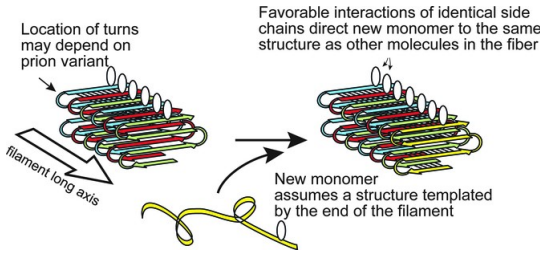

FIGURE 20.15 **Prion amyloid structure can be templated by the structure of molecules in the filament**. As a new monomer joins the filament interactions of side chains at the end of the filament with those of the new monomer force the new monomer to assume the same structure as the rest of the monomers in the filament. Different filaments may have different locations of the folds (different turn locations) and different extents of the β-sheet structure; however, this information is transmitted to new monomers joining the filament.[404,405]

HET-s Prion Amyloid Structure

Consistent with its forming only a single prion variant *in vivo*, the HET-s protein prion domain forms a very well-defined amyloid structure *in vitro*. This unique structure thus produces very sharp cross peaks in solid-state NMR, making possible the determination of its detailed structure.[320,395] This structure is a β helix, with two turns of the helix per HET-s molecule (see Fig. 20.14). Interestingly, the HET-s prion domain includes two imperfect direct repeat sequences, and these repeats are aligned in the β helix structure, producing an effect somewhat similar to the parallel in-register architecture of the yeast prions. How this structure is uniquely specified and how interaction of the HET-s amyloid and soluble HET-S produces cell death are important remaining problems.

[URE3] and [PSI+] Are Diseases of Yeast

Although mammalian prion infections are uniformly fatal, the yeast prions are often compatible with robust growth. This, and the [Het-s] prion carrying out a normal function in *Podospora anserina*, led to the suggestion that yeast prions could benefit their host. Reports of such benefits of [PSI+] and [URE3] have not been reproducible (reviewed in Ref.[404]). However, because the Sup35 of some species other than *S cerevisiae* can form prions,[59,233,336] it was assumed that prion-forming ability is selected by evolution and must therefore benefit the host,[355] a notion discredited by the finding that prion-forming ability of Ure2p and Sup35p is not conserved, even within *Saccharomyces*.[115,119,333] Similarly, the partial conservation of sequences in the prion domains of Sup35p and Ure2p was interpreted to mean that prion-forming ability was selected by evolution,[180] but sequence conservation is not needed for conserving prion-forming ability.[329,330] Therefore, sequences in the prion domains must be conserved for another reason, namely, the non-prion functions of these domains. The N-terminal domain of Sup35p of yeast and humans interacts with the poly(A) binding protein and poly(A) shortening enzymes to assist in general mRNA turnover.[79,153,188,189] The Ure2p prion domain protects the protein from rapid degradation and thus is necessary for proper nitrogen regulation.[351] These domains are preserved for their normal functions, not for prion formation.

Several approaches have been taken to evaluate whether yeast prions are diseases or beneficial. The premise that [PSI+] and [URE3] are generally not harmful to their host has been disproven by the finding that lethal or severely detrimental variants of [PSI+] are actually more common than the usually studied mild variants.[273] These “killer [PSI+]” variants probably inactivate nearly all of the essential Sup35p and are not found in the usual selection procedure because the cells are killed. [URE3] variants that severely impair growth in strains whose growth is not slowed by *ure2Δ* are also common and must be owing to a toxic effect of the Ure2p amyloid.[273]

Yeast prions are infectious and can be very stable. Moreover, they arise *de novo* in about one in 10^6 cells, thus no substantial population can be isolated from them. If they were advantageous to yeast, they would surely be often found in the wild. A survey of 70 wild yeast strains failed to turn up any strains carrying either [URE3] or [PSI+], indicating that both are a net disadvantage to the host. In contrast, the 2 μ DNA plasmid, the L-BC dsRNA viruses, and the 20S and 23S ssRNA replicons were found in 38, 8, 14, and 1 strains, respectively, although none of these selfish infectious nucleic acids encode anything but proteins to promote their own duplication and at least the 2 μ DNA plasmid is known to be detrimental.[285] A later study likewise found a very low incidence of [PSI+].[175] These data show that the *most mild* forms of [URE3], [PSI+], and [PIN+] produce a >1% growth/survival net detriment.[209] The more common toxic/lethal forms of these prions are devastating to the host. In contrast, the [Het-s] prion is found in 92% of wild *Podospora* strains carrying the *het-s* allele, consistent with it benefiting its host.[96] It appears clear that the yeast prions [URE3], [PSI+], and [PIN+] are diseases, like their mammalian counterparts.

Chaperone Involvement in Prion Propagation

Chernoff found that either overproduction of Hsp104p or deletion of the *HSP104* gene resulted in the inability of cells to propagate [PSI+][58,60] (Fig. 20.16). This finding was important as supporting the prion model for [PSI+], in opening the study of chaperones in prion phenomena, and providing a potential pathway to treatment of prion diseases. Hsp104 is necessary for the propagation of all known amyloid-based yeast prions. Normal levels of Hsp104 promote [PSI+] stability by breaking large filaments into smaller ones, providing new seeds for propagation and infection.[201,230,263,289,328,382,386] Hsp104 works with Hsp70s, nucleotide exchange factors (eg, Fes1) and Hsp40s in this activity.[167,312,412] A single molecule extracted from the middle of the filament is fed through the central pore of the Hsp104 monomer and has a chance to renature[382] (reviewed in Ref.[411]; Fig. 20.16). The mechanism of [PSI+] curing by Hsp104 overproduction is unclear[271]; however, the N-terminal domain of Hsp104 is necessary for the curing but dispensable for prion propagation, indicating that these are two distinct

activities.[194]

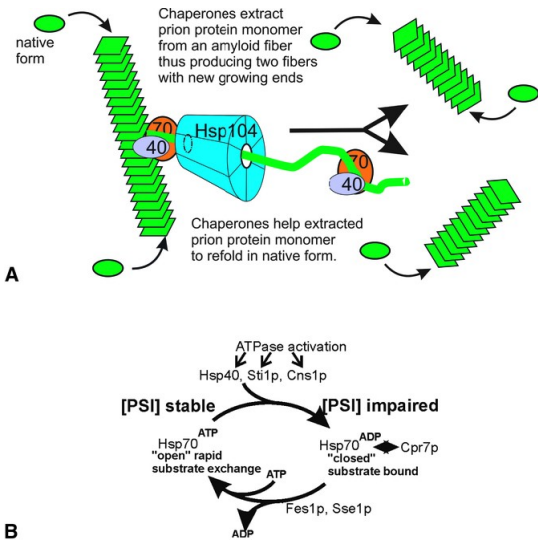

FIGURE 20.16 **A:** Role of chaperones Hsp104, Hsp70, and Hsp40 in propagation of the prions of *Saccharomyces cerevisiae*. Hsp104 is necessary for [PSI+] propagation[60,62] and for other prions (see text) for breaking filaments to form new seeds,[83,201,230,289] as are the cytoplasmic Hsp70s.[202] Hsp40s are also critical for yeast prion propagation.[182,363] Because Hsp104 cooperates with Hsp70 and Hsp40 in disaggregation,[167,382] it is proposed that they cooperate in extracting monomers from filaments, thereby breaking the fibers.[194] Overproduction of Hsp104 cures the [PSI+] prion by a different, but unknown, mechanism. Normal levels of Hsp104 are needed for [PSI+] propagation, because it has a role in breaking up aggregates to ensure that each daughter cell gets at least one aggregate. **B:** The bound ATP/ADP status of Hsp70 affects prion propagation as indicated (reviewed in Ref.[349]).

Hsp70s are important in prion propagation, with the cytoplasmic soluble Hsp70s (Ssa1,2,3,4) helping Hsp104 in propagating prions[202] (reviewed by Ref.[349]) and the ribosomal Hsp70 (Ssb1,2) antagonizing[61,213] (see below). Hsp40s play an important role in prion propagation and are believed to be the substrate-specifying component of the Hsp104-Hsp70-Hsp40 apparatus.[27,228,384] Sis1 is needed for propagation of [PSI+], [URE3], and [PIN+],[182] while Swa2 is specifically needed by [URE3].[384]

Hsp90s are also important in prion propagation, being involved in the Hsp104 overproduction curing of [PSI+][280,311] and, with their cochaperones, affecting variants of [PIN+].[238] Cpr7, a cochaperone of Hsp90, has been found necessary for the propagation of [URE3],[232] while overproduction of another Hsp90 cochaperone, Tah1, eliminates [URE3].[307] Cpr7 and Sti1, another Hsp90 cochaperone, also affect [PSI+] propagation, but both also interact with Hsp70 affecting its ATP/ADP balance, and this is the means by which they affect [PSI+].[199]

Sse1, the *S cerevisiae* version of mammalian p110, is an Hsp70 family member with a nucleotide exchange activity for Hsp70s. Overproduction or deficiency of Sse1 was found to cure [URE3-1],[229] while generation of the [PSI+] prion varied directly with levels of Sse1.[138,332] Sse1is reported to have a disaggregase activity similar to Hsp104 in requiring cooperation with Hsp70s and Hsp40s.[354] Deleting Fes1 (a nucleotide exchange factor) antagonizes [PSI+][199] and overexpressing it cures [URE3].[229]

Other cellular components also affect prion propagation (see also antiprion systems in the next section). The ubiquitin system is important in curing of [PSI+] by overproduction of Hsp104,[5,63] and the cytoskeletal protein Sla1p assists prion propagation.[13]

Antiprion Systems in *Saccharomyces cerevisiae*

Since [URE3] and [PSI+] are a net detriment to cells, it is not surprising that there is an array of cellular systems able to cure most variants, block their transmission from other cells, lower the frequency of prions arising, and limit the pathology produced by those prion variants that evade the other three types of barrier (Fig. 20.17). We consider an “antiprion system” to be components that cure prions without overexpression or deficiency of anything, an activity in normal cells.

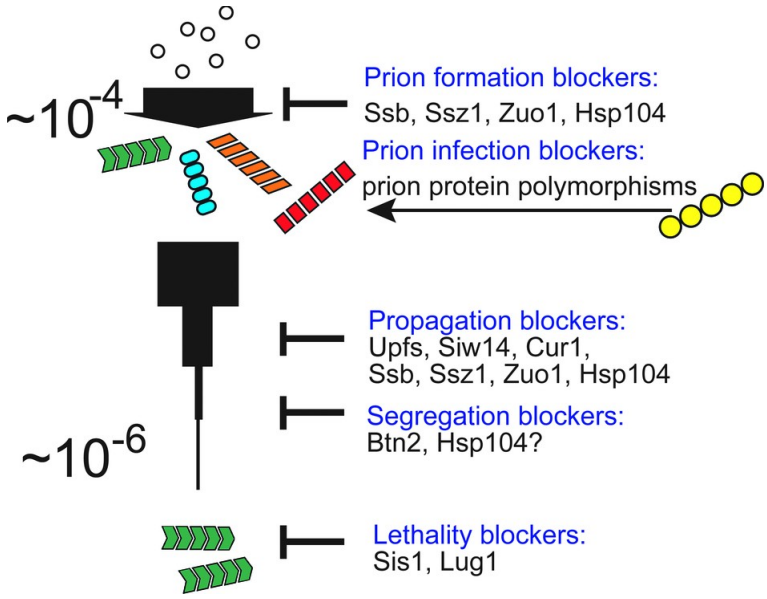

FIGURE 20.17 Antiprion systems in *S cerevisiae* partially block prion infection from another strain, block prion generation, cure most prions arising, and prevent pathology effects of those prions that survive the other three mechanisms (Adapted by permission from Springer: Wickner RB, Edskes HK, Son M, et al. Innate immunity to prions: anti-prion systems turn a tsunami of prions into a slow drip. *Curr Genet*. 2021;67(6):833-847.)

An Inter- or Intraspecies Barrier to Prion Transmission

Small differences in prion protein sequence between donor and recipient, sometimes even a single amino acid change, can partially or completely block transmission of a prion infection. Such blocking differences are found in the prion domains of Ure2p and Sup35p between mating-compatible Saccharomyces species, or even among wild strains of *S cerevisiae*.[17,55,121] The rate of sequence variation during evolution is much faster for the prion domains of Ure2p and Sup35p, than for the rest of these molecules,[121,313] in part because the protection afforded by these mutations are protective against a prion infection.[17] The polymorphism of the prion domain sequences serves as a protection against infection and thus is an antiprion system.

The degree of transmission blockage is sharply dependent on which prion variant is examined.[17,119] Prion strains can carry a mixture of variants, as was shown using the intraspecies barrier for [PSI+]. Extensive subcloning of a single [PSI+] isolate produced four distinct prion variants according to their transmission efficiency to three strains with different Sup35p prion domain sequences. Each of these variants, when again extensively grown and subcloned, gave rise to the same four variants. This phenomenon shows that prions mutate and segregate, both in the absence of any selective pressure.[16]

Btn2 and Cur1 Cure Most [URE3] Isolates Arising in Their Absence

Overproduction of Btn2 cures [URE3] by collecting the amyloid filaments at one place in the cell so that progeny cells often lack any amyloid and are cured (sequestration).[227] Overproduction of Cur1 (a paralog of Btn2) also cures [URE3] but does not collect the filaments, and its means of curing is not known.[227] Both require Hsp42 for their curing action and overproduced Hsp42 also cures [URE3].[364,398] However, normal levels of Btn2 or Cur1 cure ~90% of [URE3] isolates arising initially in a *btn2*Δ *cur1*Δ strain, and thus these are "antiprion systems".[398] The [URE3] isolates curable by normal levels of Btn2 are specifically those with the lower seed numbers (fewer amyloid filaments/cell), while [URE3] variants with higher seed number are only cured by Btn2 overproduction, consistent with the sequestration model.[398] Btn2 also has a role in endosome to Golgi protein sorting,[204] which is apparently unrelated to its prion-curing activity.[29] Btn2's "sequestrase" activity is not limited to prion amyloids but works on other aggregates.[226,257]

Btn2 and Cur1 are regulated by proteasome activity. In normal cells, Btn2 and Cur1 levels are extremely low (Btn2 ~170 molecules/cell; Cur1 ~110 molecules/cell), but in proteasome assembly or subunit mutants, their levels increase ~200-fold, the most of any of >4600 yeast proteins assayed by SILAC, a mass spectrometry method.[117] This results in rapid curing of the [URE3] prion. It is suggested that proteasomes fully occupied under stress conditions "automatically" induce the sequestration of protein aggregates by not degrading Btn2.[117]

Ribosome-Associated Chaperones

The Hsp70 family chaperones Ssb1, Ssb2, and Ssz1 and the Hsp40 member Zuo1 are largely ribosome bound and work together to insure the proper folding of nascent proteins as they emerge from the ribosome.[288,428] The frequency of [PSI+] arising is elevated dramatically in *ssb1/2*, *ssz1*, or *zuo1* mutants,[6,61,213] and over half of [PSI+] isolates arising in such mutants are cured by simply restoring the normal levels of the missing protein.[361] Possibly only improperly folded Sup35 molecules can be recruited to the amyloid filaments of many [PSI+] variants. Alternatively, since both Sup35p and these chaperones are on the ribosomes, there may be a direct interaction that competes with Sup35 adding to the ends of the filaments. In addition, Ssb1/2 freed from the ribosomes by *ssz1* or *zuo1* mutations can compete with the cytoplasmic Hsp70s (Ssa proteins) and prevent proper cleavage of prion filaments by Hsp104.[213]

Antiprion Activity of the Hsp104 Disaggregase

In addition to its filament-splitting activity (with Hsp40s and Hsp70s) that is the "replication" step in generation of new heritable prion seeds, Hsp104 overproduction cures the [PSI+] prion[60] and (weakly) [URE3].[227] The latter activity requires Hsp90 and its cochaperone Sti1[280,311] and is specifically lost by mutations in Hsp104's N-terminal domain, particularly *hsp104*^{T160M}.[194] In the *hsp104*^{T160M} mutant, [PSI+] arises spontaneously at >10-fold the rate of the wild type, and many of these isolates are cured by replacing the mutant protein with normal levels of normal Hsp104.[171] The mechanism of this curing is as yet unclear.

Inositol Polyphosphates and Prion Propagation

A general screen for antiprion systems active against [PSI+] showed that half of variants arising in an *siw14*Δ strain were cured by restoring the normal gene without overexpression.[403] Siw14 is a pyrophosphatase specific for inositol 5 pyrophosphates, and the *siw14*Δ mutant has a sixfold elevated level of these compounds.[368] Most variants of [PSI+] require some inositol poly/pyrophosphates, but evidently, some [PSI+] variants requiring an elevated level of these compounds are lost when normal levels of Siw14 lower these levels.[403] Inositol poly/pyrophosphates are signaling molecules with a variety of activities, and the means by which the curing occurs is not yet clear.

Nonsense-Mediated Decay Components Upf1,2,3 Have Antiprion Activity

mRNAs with premature stop codons are recognized and degraded rapidly, a process involving Upf1,2 and 3.[181] The anti-prion screen (above) also turned up Upf1 and Upf3 as anti-[PSI+] components.[360] In any upf mutant, [PSI+] arises at >10 fold higher rate, and most of the prion isolates are cured by replacement of normal levels of the missing protein.[360] Upf1p is associated with the Sup35 aggregates *in vitro*[90] and *in vivo* in [PSI+] cells,[360] and sub-stochiometric amounts of Upf1 inhibit amyloid formation *in vitro* by Sup35,[360] suggesting that Upfs binding to the ends of filaments may interfere with their growth. Alternatively, the normal association of Sup35 with Upf1,2,3 in a complex on the ribosome may compete for monomers with the growing filaments to block prion propagation.

Sis1 Blocks [PSI+] Lethality and Lug1 Lets [URE3] Grow

Sis1 is an Hsp40 that is essential for cell growth and also is necessary for propagation of [URE3], [PIN+], and possibly [PSI+],[182] probably because it cooperates with Hsp70s and Hsp104 in the filament cleavage reaction.[167] Certain *sis1* mutants (*sis1JGF*) able to support cell growth and prion propagation make "strong" [PSI+] variants lethal to the cell[220] by allowing the filaments to leave so little soluble Sup35p that the cells cannot grow.[231]

In a transposon mutagenesis screen, *lug1*Δ mutants were found to be lethal if a mild variant of [URE3] was present but not if it was absent, implying that Lug1 prevents the lethality of this prion.[116] Lug1 is an "F-box protein," a substrate-determining subunit of a Cullin-containing ubiquitin ligase,[346] but the ubiquitinated substrates determined by Lug1 are not yet known. Several chaperones, not previously known to be involved in [URE3], were also identified in this screen.[116] Thus, it seems that cells act to reduce the burden of carrying prions whose generation or propagation they cannot block.

Perspectives

The discovery of prions of yeast and fungi resolved the debate over whether infectious proteins could exist at all and widened the scope of this concept, showing that it is not unique to a single mammalian protein. The yeast system is now being used to explore the mechanisms of amyloid and prion generation and propagation, and to devise approaches to the many heretofore intractable amyloid diseases. Likewise, studies of retroelements, RNA viruses, and DNA viruses of unicellular eukaryotes have provided extensive information about each virus group, with much of it more easily obtained because of the facile genetics of these systems.

Acknowledgments

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REFERENCES

1. Ahn HW, Tucker JM, Arribere JA, Garfinkel DJ. Ribosome biogenesis modulates Ty1 copy number control in *Saccharomyces cerevisiae*. Genetics. 2017;207:1441-1456.

2. Aigle M, Lacroute F. Genetical aspects of [URE3], a non-Mendelian, cytoplasmically inherited mutation in yeast. Mol Gen Genet. 1975;136:327-335.

3. Aksoy S, Williams S, Chang S, Richards FF. SLACS retrotransposon from *Trypanosoma brucei gambiense* is similar to mammalian LINEs. Nucleic Acids Res. 1990;18:785-792.

4. Alberti S, Halfmann R, King O, Kapila A, Lindquist S. A systematic survey identifies prions and illuminates sequence features of prionogenic proteins. Cell. 2009;137:146-158.

5. Allen KD, Chernova TA, Tennant EP, Wilkinson KD, Chernoff YO. Effects of ubiquitin system alterations on the formation and loss of a yeast prion. J Biol Chem. 2007;282(5):3004-3013.

6. Amor AJ, Castanzo DT, Delany SP, Selechnik DM, van Ooy A, Cameron DM. The ribosome-associated complex antagonizes prion formation in yeast. Prion. 2015;9:144–164.

7. Anagnostakis SL. Biological control of chestnut blight. Science. 1982;215:466-471.

8. Anagnostakis SL, Day PR. Hypovirulence conversion in *Endothia parasitica*. Phytopathology. 1979;69:1226-1229.

9. Atkins JF, O'Conner KM, Bhatt PR, Loughran G. From recoding to peptides for MHC class I immune display: enriching viral expression, virus vulnerability and virus evasion. Viruses. 2021;13:1251.

10. Atwood A, Lin J-H, Levin HL. The retrotransposon Tf1 assembles virus-like particles that contain an excess Gag relative to integrase because of a regulated degradation process. Mol Cell Biol. 1996;16:338-346.

11. Aye M, Dildine SL, Claypool JA, Jourdain S, Sandmeyer SB. A truncation mutant of the 95-kilodalton subunit of transcription factor IIIC reveals asymmetry in Ty3 integration. Mol Cell Biol. 2001;21:7839-7851.

12. Bai M, Zhou JM, Perrett S. The yeast prion protein Ure2 shows glutathione peroxidase activity in both native and fibrillar forms. J Biol Chem. 2004;279(48):50025-50030.

13. Bailleul PA, Newnam GP, Steenbergen JN, Chernoff YO. Genetic study of interactions between the cytoskeletal assembly protein Sla1 and prion—forming domain of the release factor Sup35 (eRF3) in *Saccharomyces cerevisiae*. Genetics. 1999;153:81-94.

14. Balguerie A, Dos Reis S, Ritter C, et al. Domain organization and structure-function relationship of the HET-s prion protein of *Podospora anserina*. EMBO J. 2003;22:2071-2081.

15. Ball SG, Tirtiaux C, Wickner RB. Genetic control of L-A and L-BC dsRNA copy number in killer systems of *Saccharomyces cerevisiae*. Genetics. 1984;107:199-217.

16. Bateman D, Wickner RB. The [PSI+] prion exists as a dynamic cloud of variants. PLoS Genet. 2013;9:e1003257.

17. Bateman DA, Wickner RB. [PSI+] prion transmission barriers protect *Saccharomyces cerevisiae* from infection: intraspecies 'species barriers'. Genetics. 2012;190:569-579.

18. Baxa U, Cheng N, Winkler DC, et al. Filaments of the Ure2p prion protein have a cross-beta core structure. J Struct Biol. 2005;150(2):170-179.

19. Baxa U, Speransky V, Steven AC, Wickner RB. Mechanism of inactivation on prion conversion of the *Saccharomyces cerevisiae* Ure2 protein. Proc Natl Acad Sci U S A. 2002;99:5253-5260.

20. Baxa U, Taylor KL, Wall JS, et al. Architecture of Ure2p prion filaments: the N-terminal domain forms a central core fiber. J Biol Chem. 2003;278:43717-43727.

21. Baxa U, Wickner RB, Steven AC, et al. Characterization of b-sheet structure in Ure2p1-89 yeast prion fibrils by solid state nuclear magnetic resonance. Biochemistry. 2007;46:13149-13162.

22. Beisson-Schecroun J. Incompatibilte cellulaire et interactions nucleo-cytoplasmiques dans les phenomenes de barrage chez *Podospora anserina*. Ann Genet. 1962;4:3-50.

23. Belaikova-Bethell N, Beckham C, Giddings TH, Winey M, Parker R, Sandmeyer S. Virus-like particles of the Ty3 retrotransposon assemble in association with P-body components. RNA. 2006;12:94-101.

24. Belcourt MF, Farabaugh PJ. Ribosomal frameshifting in the yeast retrotransposon Ty: tRNAs induce slippage on a 7 nucleotide minimal site. Cell. 1990;62:339-352.

25. Belew AT, Advani VM, Dinman JD. Endogenous ribosomal frameshifting signals operate as mRNA destabilizing elements through at least two molecular pathways in yeast. Nucleic Acids Res. 2011;39(7):2799-2808.

26. Benard L, Carroll K, Valle RCP, Wickner RB. The Ski7 antiviral protein is an EF1-a homolog that blocks expression of non-poly(A) mRNA in *Saccharomyces cerevisiae*. J Virol. 1999;73:2893-2900.

27. Berger SE, Nolte AN, Kamiya E, Hines JK. Three J-proteins impact Hsp104-mediated variant-specific protein elimination: a new critical role for a low-complexity domain. Curr Genet. 2020;66:51-58.

28. Berretta J, Pinskaya M, Morillon A. A cryptic unstable transcript mediates transcriptional trans-silencing of the Ty1 retrotransposon in *S. cerevisiae*. Genes Dev. 2008;22:615-626.

29. Bezsonov EE, Edskes HE, Wickner RB. Innate immunity to yeast prions: Btn2p and Cur1p curing of the [URE3] prion is prevented by 60S ribosomal protein deficiency or ubiquitin/proteasome system overactivity. Genetics. 2021;217:iyab013. doi:10.1093/genetics/iyab013

30. Blanc A, Goyer C, Sonenberg N. The coat protein of the yeast double-stranded RNA virus L-A attaches covalently to the cap structure of eukaryotic mRNA. Mol Cell Biol. 1992;12:3390-3398.

31. Blanc A, Ribas JC, Wickner RB, Sonenberg N. His154 is involved in the linkage of the *Saccharomyces cerevisiae* L-A double-stranded RNA virus *gag* protein to the cap structure of mRNAs and is essential for M1 satellite virus expression. Mol Cell Biol. 1994;14:2664-2674.

32. Boccardo G, Milne RG, Luisoni E, Lisa V, Accotto GP. Three seedborne cryptic viruses containing double-stranded RNA isolated from white clover. Virology. 1985;147:29-40.

33. Boeke JD, Sandmeyer SB. Yeast transposable elements. In: Broach JR, Pringle JR, Jones EW, eds. The Molecular and Cellular Biology of the Yeast Saccharomyces cerevisiae: Genome Dynamics, Protein Synthesis and Energetics. Cold Spring Harbor Laboratory Press; 1991:193-261.

34. Boeke JD, Stoye JP. Retrotransposons, endogenous retroviruses and the evolution of retroelements. In: Coffin JM, Hughes SH, Varmus HE, eds. Retroviruses. Cold Spring Harbor Laboratory Press; 1997.

35. Bolton EC, Coombes C, Eby Y, Cardell M, Boeke JD. Identification and characterization of critical cis-acting sequences within the yeast Ty1 retrotransposon. RNA. 2005;11:308-322.

36. Bostian KA, Elliott Q, Bussey H, Burn V, Smith A, Tipper DJ. Sequence of the preprotoxin dsRNA gene of type I killer yeast: multiple processing events produce a two-component toxin. Cell. 1984;36(3):741-751.

37. Bousset L, Beirhali H, Janin J, Melki R, Morera S. Structure of the globular region of the prion protein Ure2 from the yeast *Saccharomyces cerevisiae*. Structure. 2001;9:39-46.

38. Brachmann A, Baxa U, Wickner RB. Prion generation *in vitro*: amyloid of Ure2p is infectious. EMBO J. 2005;24:3082-3092.

39. Bradley ME, Edskes HK, Hong JY, Wickner RB, Liebman SW. Interactions among prions and prion "strains" in yeast. Proc Natl Acad Sci U S A. 2002;99(Suppl 4):16392-16399.

40. Bradshaw VA, McEntee K. DNA damage activates transcription and transposition of yeast Ty retrotransposons. Mol Gen Genet. 1989;218:465-474.

41. Brennan VE, Field L, Cizdziel P, Bruenn JA. Sequences at the 3′ ends of yeast viral dsRNAs: proposed transcriptase and replicase initiation sites. Nucleic Acids Res. 1981;9(16):4007-4021.

42. Brierley I, Jenner AJ, Inglis SC. Mutational analysis of the "slippery-sequence" component of a coronavirus ribosomal frameshifting signal. J Mol Biol. 1992;227:463-479.

43. Brown JT, Bai X, Johnson AW. The yeast antiviral proteins Ski2p, Ski3p and Ski8p exist as a complex in vivo. RNA. 2000;6:449-457.

44. Bruce ME. Scrapie strain variation and mutation. Br Med Bull. 1993;49:822-838.

45. Cam HP, Noma K-I, Ebina H, Levin HL, Grewal SIS. Host genome surveillance for retrotransposons by transposon-derived proteins. Nature. 2008;451:431-436.

46. Caston JR, Trus BL, Booy FP, Wickner RB, Wall JS, Steven AC. Structure of L-A virus: a specialized compartment for the transcription and replication of double-stranded RNA. J Cell Biol. 1997;138:975-985.

47. Chakrabortee S, Kayatekin C, Newby GA, Mendillo ML, Landcaster A, Lindquist S. Luminidependens (LD) is an Arabidopsis protein with prion behavior. Proc Natl Acad Sci U S A. 2016;113:6065-6070.

48. Chakravarty AK, Smejkal T, Itakura AK, Garcia DM, Jarosz DF. A non-amyloid prion particle that activates a heritable gene expression program. Mol Cell. 2020;77:251-265.

49. Chalker DL, Sandmeyer SB. Ty3 integrates within the region of RNA polymerase II transcription initiation. Genes Dev. 1992;6:117-128.

50. Chamorro M, Parkin N, Varmus HE. An RNA pseudoknot and an optimal heptameric shift site are required for highly efficient ribosomal frameshifting on a retroviral messenger RNA. Proc Natl Acad Sci U S A. 1992;89:713-717.

51. Chapman KB, Boeke JD. Isolation and characterization of the gene encoding yeast debranching enzyme. Cell. 1991;65:483-492.

52. Chapman KB, Bystrom AS, Boeke JD. Initiator methionine tRNA is essential for Ty1 transposition in yeast. Proc Natl Acad Sci U S A. 1992;89:3236-3240.

53. Checkley MA, Nagashima K, Lockett SJ, Nyswanter KM, Garfinkel DJ. P-body components are required for Ty1 retrotransposition during assembly of retrotransposition-competent virus-like particles. Mol Cell Biol. 2010;30:382-398.

54. Chen B, Gao S, Choi GH, Nuss DL. Extensive alteration of fungal gene transcript accumulation and elevation of G-protein-regulated cAMP levels by a virulence-attenuating hypovirus. Proc Natl Acad Sci U S A. 1996;93:7996-8000.

55. Chen B, Newnam GP, Chernoff YO. Prion species barrier between the closely related yeast proteins is detected despite coaggregation. Proc Natl Acad Sci U S A. 2007;104(8):2791-2796.

56. Chen B, Thurber KR, Shewmaker F, Wickner RB, Tycko R. Measurement of amyloid fibril mass-per-length by tilted-beam transmission electron microscopy. Proc Natl Acad Sci U S A. 2009;106:14339-14344.

57. Cheng Z, Menees TM. RNA branching and debranching in the yeast retrovirus-like element Ty1. Science. 2004;303:240-243.

58. Chernoff YO, Derkach IL, Inge-Vechtomov SG. Multicopy SUP35 gene induces de-novo appearance of psi-like factors in the yeast *Saccharomyces cerevisiae*. Curr Genet. 1993;24:268-270.

59. Chernoff YO, Galkin AP, Lewitin E, Chernova TA, Newnam GP, Belenkiy SM. Evolutionary conservation of prion-forming abilities of the yeast Sup35 protein. Mol Microbiol. 2000;35:865-876.

60. Chernoff YO, Lindquist SL, Ono B-I, Inge-Vechtomov SG, Liebman SW. Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+]. Science. 1995;268:880-884.

61. Chernoff YO, Newnam GP, Kumar J, Allen K, Zink AD. Evidence for a protein mutator in yeast: role of the Hsp70-related chaperone Ssb in formation, stability and toxicity of the [PSI+] prion. Mol Cell Biol. 1999;19:8103-8112.

62. Chernoff YO, Ono B-I. Dosage-dependent modifiers of PSI-dependent omnipotent suppression in yeast. In: Brown AJP, Tuite MF, McCarthy JEG, eds. Protein Synthesis and Targeting in Yeast. Springer-Verlag; 1992:101-107.

63. Chernova TA, Allen KD, Wesolowski LM, Shanks JR, Chernoff YO, Wilkinson KD. Pleiotropic effects of Ubp6 loss on drug sensitivities and yeast prion are due to depletion of the free ubiquitin pool. J Biol Chem. 2003;278:52102-52115.

64. Chiba S, Jamal A, Suzuki N. First evidence for internal ribosome entry sites in diverse fungal virus genomes. MBio. 2018;9:e02350-17.

65. Choi GH, Chen B, Nuss DL. Virus-mediated or transgenic suppression of a G protein a subunit and attenuation of fungal virulence. Proc Natl Acad Sci U S A. 1995;92:305-309.

66. Choi GH, Larson TG, Nuss DL. Molecular analysis of the laccase gene from the chestnut blight fungus and selective suppression of its expression in an isogenic hypovirulent strain. Mol Plant Microbe Interact. 1992;5:119-128.

67. Choi GH, Nuss DL. Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. Science. 1992;257:800-803.

68. Choi GH, Nuss DL. A viral gene confers hypovirulence-associated traits to the chestnut blight fungus. EMBO J. 1992;11:473-477.

69. Choi GH, Shapira R, Nuss DL. Cotranslational autoproteolysis involved in gene expression from a double-stranded RNA genetic element associated with hypovirulence of the chestnut blight fungus. Proc Natl Acad Sci U S A. 1991;88:1167-1171.

70. Ciriacy M, Freidel K, Lohning C. Characterization of trans-acting mutations affecting Ty and Ty-mediated transcription in *Saccharomyces cerevisiae*. Curr Genet. 1991;20:441-448.

71. Clare J, Farabaugh PJ. Nucleotide sequence of a yeast Ty element: evidence for an unusual mechanism of gene expression. Proc Natl Acad Sci U S A. 1985;82:2828-2833.

72. Clark DJ, Bilanchone VW, Haywood LJ, Dildine SL, Sandmeyer SB. A yeast sigma composite element, Ty3, has properties of a retrotransposon. J Biol Chem. 1988;263:1413-1423.

73. Company M, Errede B. Identification of a Ty1 regulatory sequence responsive to *STE7* and *STE12*. Mol Cell Biol. 1988;8:2545-2554.

74. Conte DJ, Barber E, Banerjee M, Garfinkel DJ, Curcio MJ. Posttranslational regulation of Ty1 retrotransposition by mitogen-activated protein kinase Fus3. Mol Cell Biol. 1998;18:2502-2513.

75. Conte DJ, Curcio MJ. Fus3 controls Ty1 transpositional dormancy through the invasive growth MAPK pathway. Mol Microbiol. 2000;35:415-427.

76. Coombes CE, Boeke JD. An evaluation of detection methods for large lariat RNAs. RNA. 2005;11:323-331.

77. Cooper A, Bussey H. Characterization of the yeast KEX1 gene product: a carboxypeptidase involved in processing secreted precursor proteins. Mol Cell Biol. 1989;9(6):2706-2714.

78. Cooper TG. Transmitting the signal of excess nitrogen in *Saccharomyces cerevisiae* from the Tor proteins to the GATA factors: connecting the dots. FEMS Microbiol Rev. 2002;26:223-238.

79. Cosson B, Couturier A, Chabelskaya S, et al. Poly(A)-binding protein acts in translation termination via eukaryotic release factor 3 interaction and does not influence [PSI+] propagation. Mol Cell Biol. 2002;22:3301-3315.

80. Cottee MA, Beckwith SL, Letham SC, et al. Structure of a Ty1 restriction factor reveals the molecular basis of transposition copy number control. Nat Commun. 2021;12(1):5590.

81. Coustou V, Deleu C, Saupe S, Begueret J. The protein product of the *het-s* heterokaryon incompatibility gene of the fungus *Podospora anserina* behaves as a prion analog. Proc Natl Acad Sci U S A. 1997;94:9773-9778.

82. Cox BS. PSI, a cytoplasmic suppressor of super-suppressor in yeast. Heredity. 1965;20:505-521.

83. Cox BS, Ness F, Tuite MF. Analysis of the generation and segregation of propagons: entities that propagate the [PSI+] prion in yeast. Genetics. 2003;165:23-33.

84. Craven MG, Pawlyk DM, Choi GH, Nuss DL. Papain-like protease p29 as a symptom determinant encoded by a hypovirulence-associated virus of the chestnut blight fungus. J Virol. 1993;67:6513-6521.

85. Curcio MJ. Border collies of the genome: domestication of an autonomous retrovirus-like transposon. Curr Genet. 2019;65:71-78.

86. Curcio MJ, Garfinkel DJ. Single-step selection for Ty1 element retrotransposition. Proc Natl Acad Sci U S A. 1991;88:936-940.

87. Curcio MJ, Garfinkel DJ. Posttranslational control of Ty1 retrotransposition occurs at the level of protein processing. Mol Cell Biol. 1992;12:2813-2825.

88. Curcio MJ, Kenny AE, Moore S, et al. S-phase checkpoint pathways stimulate the mobility of the retrovirus-like transposon Ty1. Mol Cell Biol. 2007;27:8874-8885.

89. Curcio MJ, Sanders NJ, Garfinkel DJ. Transcriptional competence and transcription of endogenous Ty elements in *Saccharomyces cerevisiae*: implications for regulation of transposition. Mol Cell Biol. 1988;8:3571-3581.

90. Czaplinski K, Ruiz-Echevarria MJ, Paushkin SV, et al. The surveillance complex interacts with the translation release factors to enhance termination and degrade aberrant mRNAs. Genes Dev. 1998;12:1665-1677.

91. Dai J, Xie W, Brady TL, Gao J, Voytas DF. Phosphorylation regulates integration of the yeast Ty5 retrotransposon into heterochromatin. Mol Cell. 2007;27:289-299.

92. Dalstra HJP, Swart K, Debets AJM, Saupe SJ, Hoekstra RF. Sexual transmission of the [Het-s] prion leads to meiotic drive in *Podospora anserina*. Proc Natl Acad Sci U S A. 2003;100:6616-6621.

93. Daskalov A, Mammeri NE, Lends A, et al. Structures of pathological and functional amyloids and prions, a solid-state NMR perspective. Front Mol Neurosci. 2021;14:670513.

94. Daskalov A, Paoletti M, Ness F, Saupe SJ. Genomic clustering and homology between HET-S and the NWD2 STAND protein in various fungal genomes. PLoS One. 2012;7:e34854.

95. Day PR, Dodds JA, Elliston JE, Jaynes RA, Anagnostakis SL. Double-stranded RNA in *Endothia parasitica*. Phytopathology. 1977;67:1393-1396.

96. Debets AJ, Dalstra HJ, Slakhorst M, Koopmanschap B, Hoekstra RF, Saupe SJ. High natural prevalence of a fungal prion. Proc Natl Acad Sci U S A. 2012;109:10432-10437.

97. Deleu C, Clave C, Begueret J. A single amino acid difference is sufficient to elicit vegetative incompatibility in the fungus *Podospora anserina*. Genetics. 1993;135:45-52.

98. Derkatch IL, Bradley ME, Hong JY, Liebman SW. Prions affect the appearance of other prions: the story of *[PIN]*. Cell. 2001;106:171-182.

99. Derkatch IL, Bradley ME, Zhou P, Chernoff YO, Liebman SW. Genetic and environmental factors affecting the *de novo* appearance of the [*PSI*+] prion in *Saccharomyces cerevisiae*. Genetics. 1997;147:507-519.

100. Derkatch IL, Chernoff YO, Kushnirov VV, Inge-Vechtomov SG, Liebman SW. Genesis and variability of [*PSI*] prion factors in *Saccharomyces cerevisiae*. Genetics. 1996;144:1375-1386.

101. Derr LK, Strathern JN, Garfinkel DJ. RNA-mediated recombination in *S. cerevisiae*. Cell. 1991;67:355-364.

102. Diaz-Avalos R, King CY, Wall JS, Simon M, Caspar DLD. Strain-specific morphologies of yeast prion amyloids. Proc Natl Acad Sci U S A. 2005;102:10165-10170.

103. Dignard D, Whiteway M, Germain D, Tessier D, Thomas DY. Expression in yeast of a cDNA copy of the K2 killer toxin gene. Mol Gen Genet. 1991;227:127-136.

104. Dihanich M, Van Tuinen E, Lambris JD, Marshallsay B. Accumulation of viruslike particles in a yeast mutant lacking a mitochondrial pore protein. Mol Cell Biol. 1989;9:1100-1108.

105. Dinman JD, Icho T, Wickner RB. A -1 ribosomal frameshift in a double-stranded RNA virus of yeast forms a gag-pol fusion protein. Proc Natl Acad Sci U S A. 1991;88(1): 174-178.

106. Dinman JD, Ruiz-Echevarria MJ, Peltz SW. Translating old drugs into new treatments: ribosomal frameshifting as a target for antiviral agents. Trends Biotechnol. 1998;16:190-196.

107. Dinman JD, Wickner RB. Ribosomal frameshifting efficiency and *gag/gag-pol* ratio are critical for yeast M1 double-stranded RNA virus propagation. J Virol. 1992;66:3669-3676.

108. Dinman JD, Wickner RB. 5S rRNA is involved in fidelity of translational reading frame. Genetics. 1995;141:95-105.

109. Dodds JA. Association of type 1 viral-like dsRNA with club-shaped particles in hypovirulent strains of *Endothia parasitica*. Virology. 1980;107:1-12.

110. Doel SM, McCready SJ, Nierras CR, Cox BS. The dominant *PNM2*– mutation which eliminates the [PSI] factor of *Saccharomyces cerevisiae* is the result of a missense mutation in the *SUP35* gene. Genetics. 1994;137:659-670.

111. Dong C, Poulter RTM, Han JS. LINE-like retrotransposition in *Saccharomyces cerevisiae*. Genetics. 2009;181:301-311.

112. Drinnenberg IA, Fink GR, Bartel DP. Compatibility with killer explains the rise of RNAi-deficient fungi. Science. 2011;333:1592.

113. Du Z, Park K-W, Yu H, Fan Q, Li L. Newly identified prion linked to the chromatin-remodeling factor Swi1 in *Saccharomyces cerevisiae*. Nat Genet. 2008;40:460-465.

114. Dutko JA, Kenny AE, Gamache ER, Curcio MJ. 5′ to 3′ mRNA decay factors colocalize with Ty1 Gag and human APOBEC3G and promote Ty1 retrotransposition. J Virol. 2010;84:5052-5066.

115. Edskes HE, Khamar HJ, Winchester C-L, et al. Sporadic distribution of prion-forming ability of Sup35p from yeasts and fungi. Genetics. 2014;198:605-616.

116. Edskes HE, Mukhamedova M, Edskes BK, Wickner RB. Hermes transposon mutagenesis shows [URE3] prion pathology prevented by a ubiquitin-targeting protein: evidence for carbon/nitrogen assimilation cross-talk and a second function for Ure2p. Genetics. 2018;209:789-800.

117. Edskes HE, Stroobant EE, DeWilde M, Bezsonov EE, Wickner RB. Proteasome control of [URE3] prion propagation by degradation of anti-prion proteins Cur1 and Btn2 in *Saccharomyces cerevisiae*. Genetics. 2021;218(1):iyab037. doi:10.1093/genetics/iyab037

118. Edskes HK, Gray VT, Wickner RB. The [URE3] prion is an aggregated form of Ure2p that can be cured by overexpression of Ure2p fragments. Proc Natl Acad Sci U S A. 1999;96:1498-1503.

119. Edskes HK, McCann LM, Hebert AM, Wickner RB. Prion variants and species barriers among *Saccharomyces* Ure2 proteins. Genetics. 2009;181:1159-1167.

120. Edskes HK, Ohtake Y, Wickner RB. Mak21p of *Saccharomyces cerevisiae*, a homolog of human CAATT-binding protein, is essential for 60S ribosomal subunit biogenesis. J Biol Chem. 1998;273:28912-28920.

121. Edskes HK, Wickner RB. Conservation of a portion of the *S. cerevisiae* Ure2p prion domain that interacts with the full - length protein. Proc Natl Acad Sci U S A. 2002;99 (Suppl 4):16384-16391.

122. Eibel H, Philippsen P. Preferential integration of yeast transposable element Ty into a promoter region. Nature. 1984;307:386-388.

123. Eichinger DJ, Boeke JD. A specific terminal structure is required for Ty1 transposition. Genes Dev. 1990;4:324-330.

124. Enebak SA, Hillman BI, MacDonald WL. A hypovirulent isolate of *Cryphonectria parasitic*a with multiple, genetically unique dsRNA segments. Mol Plant Microbe Interact. 1994;7:590-595.

125. Errede B. MCM1 binds to a transcriptional control element in Ty1. Mol Cell Biol. 1993;13:57-62.

126. Errede B, Cardillo TS, Sherman F, Dubois E, Deschamps J, Wiame JM. Mating signals control expression of mutations resulting from insertion of a transposable repetitive element adjacent to diverse yeast genes. Cell. 1980;22:427-436.

127. Errede B, Cardillo TS, Wever G, Sherman F. Studies on transposable elements in yeast. I. ROAM mutations causing increased expression of yeast genes: their activation by signals directed toward conjugation functions and their formation by insertion of Ty1 repetitive elements. Cold Spring Harb Symp Quant Biol. 1981;45:593-602.

128. Esteban LM, Fujimura T, Garcia-Cuellar M, Esteban R. Association of yeast viral 23S RNA with its putative RNA-dependent RNA polymerase. J Biol Chem. 1994;269:29771-29777.

129. Esteban LM, Rodriguez CN, Esteban R. T double-stranded RNA (dsRNA) sequence reveals that T and W dsRNAs form a new RNA family in Saccharomyces cerevisiae. Identification of 23 S RNA as the single-stranded form of T dsRNA. J Biol Chem. 1992;267(15):10874-10881.

130. Esteban R, Fujimura T. Launching the yeast 23S RNA Narnavirus shows 5′ and 3′ cis-acting signals for replication. Proc Natl Acad Sci U S A. 2003;100:2568-2573.

131. Esteban R, Fujimura T, Wickner RB. Internal and terminal cis-acting sites are necessary for in vitro replication of the L-A double-stranded RNA virus of yeast. EMBO J. 1989;8(3):947-954.

132. Esteban R, Vega L, Fujimura T. Launching of the yeast 20S RNA narnavirus by expressing the genomic or antigenomic viral RNA *in vivo*. J Biol Chem. 2005;280:33725-33734.

133. Esteban R, Vega L, Fujimura T. 20S RNA narnavirus defies the antiviral activity of *SKI1/XRN1* in *Saccharomyces cerevisiae*. J Biol Chem. 2008;283:25812-25820.

134. Esteban R, Wickner RB. Three different M1 RNA-containing viruslike particle types in Saccharomyces cerevisiae: in vitro M1 double-stranded RNA synthesis. Mol Cell Biol. 1986;6(5):1552-1561.

135. Esteban R, Wickner RB. A new non-mendelian genetic element of yeast that increases cytopathology produced by M1 double-stranded RNA in ski strains. Genetics. 1987;117(3):399-408.

136. Esteban R, Wickner RB. A deletion mutant of L-A double-stranded RNA replicates like M1 double-stranded RNA. J Virol. 1988;62(4):1278-1285.

137. Fahima T, Kazmierczak P, Hansen DR, Pfeifer P, Alfen NKV. Membrane-associated replication of an unencapsidated double-stranded RNA of the fungus, *Cryphonectria parasitica*. Virology. 1993;195:81-89.

138. Fan Q, Park K-W, Du Z, Morano KA, Li L. The role of Sse1 in the de novo formation and variant determination of the [PSI+] prion. Genetics. 2007;177:1583-1593.

139. Farabaugh PJ, Fink GR. Insertion of the eukaryotic transposable element Ty1 creates a 5 base pair duplication. Nature. 1980;286:352-356.

140. Farabaugh PJ, Zhao H, Vimaladithan A. A novel programmed frameshift expresses the *POL3* gene of retrotranposon Ty3 of yeast: frameshifting without tRNA slippage. Cell. 1993;74:93-103.

141. Fujimura T, Esteban R. Interactions of the RNA polymerase with the viral genome at the 5′ and 3′ ends contribute to 20S RNA narnavirus persistence in yeast. J Biol Chem. 2007;282:19011-19019.

142. Fujimura T, Esteban R. Cap-snatching mechanism in yeast L-A double-stranded RNA virus. Proc Natl Acad Sci U S A. 2011;108:17667-17671.

143. Fujimura T, Esteban R. Cap snatching of yeast L-A double-stranded RNA virus can operate in trans and requires viral polymerase actively engaging in transcription. J Biol Chem. 2012;287:12797-12804.

144. Fujimura T, Esteban R. Diphosphates at the 5′ end of the positive strand of yeast L-A double-stranded RNA virus as a molecular self-identity tag. Mol Microbiol. 2016;102:71-80.

145. Fujimura T, Esteban R, Esteban LM, Wickner RB. Portable encapsidation signal of the L-A double-stranded RNA virus of *S. cerevisiae*. Cell. 1990;62(4):819-828.

146. Fujimura T, Esteban R, Wickner RB. In vitro L-A double-stranded RNA synthesis in virus-like particles from Saccharomyces cerevisiae. Proc Natl Acad Sci U S A. 1986;83(12):4433-4437.

147. Fujimura T, Ribas JC, Makhov AM, Wickner RB. Pol of gag-pol fusion protein required for encapsidation of viral RNA of yeast L-A virus. Nature. 1992;359:746-749.

148. Fujimura T, Solorzano A, Esteban R. Native replication intermediates of the yeast 20S RNA virus have a single-stranded RNA backbone. J Biol Chem. 2005;280:7398-7406.

149. Fujimura T, Wickner RB. Gene overlap results in a viral protein having an RNA binding domain and a major coat protein domain. Cell. 1988;55(4):663-671.

150. Fujimura T, Wickner RB. Replicase of L-A virus-like particles of Saccharomyces cerevisiae. In vitro conversion of exogenous L-A and M1 single-stranded RNAs to double-stranded form. J Biol Chem. 1988;263(1):454-460.

151. Fujimura T, Wickner RB. Reconstitution of template-dependent in vitro transcriptase activity of a yeast double-stranded RNA virus. J Biol Chem. 1989;264(18):10872-10877.

152. Fujimura T, Wickner RB. Interaction of two cis sites with the RNA replicase of the yeast L-A virus. J Biol Chem. 1992;267(4):2708-2713.

153. Funakoshi Y, Doi Y, Hosoda N, et al. Mechanism of mRNA deadenylation: evidence for a molecular interplay between translation termination factor eRF3 and mRNA deadenylases. Genes Dev. 2007;21:3135-3148.

154. Gabriel A, Yen TJ, Schwartz DC, et al. A rapidly rearranging retrotransposon within the miniexon gene locus of *Crithidia fasciculata*. Mol Cell Biol. 1990;10:615-624.

155. Gao J, Chau S, Chowdhury F, et al. Meiotic viral attenuation through an ancestral apoptotic pathway. Proc Natl Acad Sci U S A. 2019;116:16454-16462.

156. Gao J, Chau S, Meneghini MD. Viral attenuation by endonuclease G during yeast gametogenesis: insights into ancestral roles of programmed cell death? Microb Cell. 2019;7:32-35.

157. Gao S, Nuss DL. Distinct roles for two G-protein a subunits in fungal virulence, morphology and reproduction revealed by targeted gene disruption. Proc Natl Acad Sci U S A. 1996;93:14122-14127.

158. Garcia-Cuellar MP, Esteban LM, Fujimura T, Rodriguez-Cousino N, Esteban R. Yeast viral 20S RNA is associated with its cognate RNA-dependent RNA polymerase. J Biol Chem. 1995;270:20084-20089.

159. Garcia-Cuellar MP, Esteban R, Fujimura T. RNA-dependent RNA polymerase activity associated with the yeast viral p91/20S RNA ribonucleoprotein complex. RNA. 1997;3:27-36.

160. Garfinkel DJ, Hedge A-M, Youngren SD, Copeland TD. Proteolytic processing of *pol-TYB* proteins from the yeast retrotransposon Ty1. J Virol. 1991;65:4573-4581.

161. Garfinkel DJ, Nyswaner K, Wang J, Cho J-Y. Post-transcriptional cosuppression of Ty1 retrotransposition. Genetics. 2003;165:83-99.

162. Garfinkel DJ, Tucker JM, Saha A, et al. A self-encoded capsid derivative restricts Ty1 retrotranposition in *Saccharomyces*. Curr Genet. 2016;62:321-329.

163. Garnepudi VR, Zhao C, Beeler T, Dunn T. Serine palmitoyltransferase (*scs1/lcb2*) mutants have elevated copy number of the L-A dsRNA virus. Yeast. 1997;13:299-304.

164. Garvik B, Haber JE. New cytoplasmic genetic element that controls 20S RNA synthesis during sporulation in yeast. J Bacteriol. 1978;134:261-269.

165. Georgopoulos DE, Hannig EM, Leibowitz MJ. Sequence of the M1-2 region of killer virus double-stranded RNA. Basic Life Sci. 1986;40(203):203-213.

166. Glover JR, Kowal AS, Shirmer EC, Patino MM, Liu J-J, Lindquist S. Self-seeded fibers formed by Sup35, the protein determinant of [*PSI*+], a heritable prion-like factor of *S. cerevisiae*. Cell. 1997;89:811-819.

167. Glover JR, Lindquist S. Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell. 1998;94(1):73-82.

168. Goncalves AP, Heller J, Daskalov A, Videira A, Glass NL. Regulated forms of cell death in fungi. Front Microbiol. 2018;8:1837.

169. Goodwin TJD, Ormandy JE, Poulter RTM. L1-like non-LTR retrotransposons in the yeast *Candida albicans*. Curr Genet. 2001;39:83-91.

170. Goodwin TJD, Poulter RTM. The diversity of retrotransposons in the yeast *Cryptococcus neoformans*. Yeast. 2001;18:865-880.

171. Gorkovskiy A, Reidy M, Masison DC, Wickner RB. Hsp104 at normal levels cures many [PSI+] variants in a process promoted by Sti1p, Hsp90 and Sis1p. Proc Natl Acad Sci U S A. 2017;114:E4193-E4202.

172. Greenall A, Williams ES, Martin KA, et al. Hip3 interacts with the HIRA proteins Hip2 and Slm9 and is required for transcriptional silencing and accurate chromosome segregation. J Biol Chem. 2006;281:8732-8739.

173. Gumna J, Andrzejewska-Romanowska A, Garfinkel DJ, Pachulska-Wieczorek K. RNA binding properties of the Ty1 LTR-retrotransposon Gag protein. Int J Mol Sci. 2021;22:9103.

174. Guo LH, Sun L, Chiba S, Araki H, Suzuki N. Coupled termination/reinitiation for translation of the downstream open reading frame B of the prototypic hypovirus CHV1-EP713. Nucleic Acids Res. 2009;37:3645-3659.

175. Halfmann R, Jarosz DF, Jones SK, Chang A, Lancster AK, Lindquist S. Prions are a common mechanism for phenotypic inheritance in wild yeasts. Nature. 2012;482:363-368.

176. Hammond TM, Andrewski MD, Roossinck MJ, Keller MP. *Aspergillus* mycoviruses are targets and suppressors of RNA silencing. Eukaryot Cell. 2008;7:350-357.

177. Hansen DR, Alfen NKV, Gillies K, Powell WA. Naked dsRNA association with hypovirulence in *Endothia parasitica* is packaged in fungal vesicles. J Gen Virol. 1985;66:2605-2614.

178. Hansen LJ, Chalker DL, Sandmeyer SB. Ty3, a yeast retrotransposon associated with tRNA genes, has homology to animal retroviruses. Mol Cell Biol. 1988;8:5245-5256.

179. Hao F, Wu M, Li G. Molecular characterization and geographic distribution of a Mymonavirus in the population of *Botrytis cinerea*. Viruses. 2018;10(8):432. doi:10.3390/v10080432

180. Harrison LB, Yu Z, Stajich JE, Dietrich FS, Harrison PM. Evolution of budding yeast prion-determinant sequences across diverse fungi. J Mol Biol. 2007;368(1):273-282. doi:10.1016/j.jmb.2007.01.070

181. He F, Jacobson A. Nonsense-mediated mRNA decay: degradation of defective transcripts is only part of the story. Annu Rev Genet. 2015;49:339-366.

182. Higurashi T, Hines JK, Sahi C, Aron R, Craig EA. Specificity of the J-protein Sis1 in the propagation of 3 yeast prions. Proc Natl Acad Sci U S A. 2008;105:16596-16601.

183. Hillman BI, Annisa A, Suzuki N. Viruses of plant-interacting fungi. Adv Virus Res. 2018;100:99-116.

184. Hillman BI, Cai G. The family narnaviridae: simples of RNA viruses. Adv Virus Res. 2013;86:149-176.

185. Hillman BI, Supyani S, Kondo H, Suzuki N. A reovirus of the fungus *Cryphonectria parasitica* that is infectious as particles and related to the coltivirus genus of animal pathogens. J Virol. 2004;78:892-898.

186. Hillman BI, Suzuki N. Virus of the chestnut blight fungus, *Cryphonectria parasitica*. Adv Virus Res. 2004;63:423-472.

187. Honda S, Eusebio-Cope A, Miyashita S, et al. Establishment of *Neurospora crassa* as a model organism for fngal virology. Nat Commun. 2020;11:5627.

188. Hoshino S, Imai M, Kobayashi T, Uchida N, Katada T. The eukaryotic polypeptide chain releasing factor (eRF3/GSPT) carrying the translation termination signal to the 3′-poly(A) tail of mRNA. J Biol Chem. 1999;274:16677-16680.

189. Hosoda N, Kobayashii T, Uchida N, et al. Translation termination factor eRF3 mediates mRNA decay through the regulation of deadenylation. J Biol Chem. 2003;278:38287-38291.

190. Hsu CL, Stevens A. Yeast cells lacking 5′-->3′ exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5′ cap structure. Mol Cell Biol. 1993;13:4826-4835.

191. Huang H, Hong JY, Burck CL, Liebman SW. Host genes that affect the target-site distribution of the yeast retrotransposon Ty1. Genetics. 1999;151:1393-1407.

192. Huang Q, Purzycka KJ, Lusvarghi S, Li D, Legrice SF, Boeke JD. Retrotransposon Ty1 RNA contains a 5′-terminal long-range pseudoknot required for efficient reverse transcription. RNA. 2013;19:320-332.

193. Huang S, Ghabrial SA. Organization and expression of the double-stranded RNA genome of *Helminthosporium victoriae* 190S virus, a totivirus infecting a plant pathogenic filamentous fungus. Proc Natl Acad Sci U S A. 1996;93:12541-12546.

194. Hung GC, Masison DC. N-terminal domain of yeast Hsp104 chaperone is dispensable for thermotolerance and prion propagation but necessary for curing prions by Hsp104 overexpression. Genetics. 2006;173:611-620.

195. Icho T, Wickner RB. The double-stranded RNA genome of yeast virus L-A encodes its own putative RNA polymerase by fusing two open reading frames. J Biol Chem. 1989;264(12):6716-6723.

196. Jacks T, Madhani HD, Masiarz FR, Varmus HE. Signals for ribosomal frameshifting in the Rous sarcoma virus gag-pol region. Cell. 1988;55:447-458.

197. Jacob-Wilk D, Turina M, Van Alfen NK. Mycovirus cryphonectria hypovirus 1 elements cofractionate with trans-Golgi network membranes of the fungal host *Cryphonectria parasitica*. J Virol. 2006;80:6588-6596.

198. Jacobs-Anderson JS, Parker R. The 3′ to 5′ degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3′ to 5′ exonucleases of the exosome complex. EMBO J. 1998;17:1497-1506.

199. Jones G, Song Y, Chung S, Masison DC. Propagation of yeast [PSI+] prion impaired by factors that regulate Hsp70 substrate binding. Mol Cell Biol. 2004;24:3928-3937.

200. Julius D, Brake A, Blair L, Kunisawa R, Thorner J. Isolation of the putative structural gene for the lysine - arginine - cleaving endopeptidase required for the processing of yeast prepro-alpha factor. Cell. 1984;37:1075-1089.

201. Jung G, Jones G, Masison DC. Amino acid residue 184 of yeast Hsp104 chaperone is critical for prion-curing by guanidine, prion propagation, and thermotolerance. Proc Natl Acad Sci U S A. 2002;99:9936-9941.

202. Jung G, Jones G, Wegrzyn RD, Masison DC. A role for cytosolic Hsp70 in yeast [PSI+] prion propagation and [PSI+] as a cellular stress. Genetics. 2000;156:559-570.

203. Kadowaki K, Halvorson HO. Appearance of a new species of ribonucleic acid during sporulation in *Saccharomyces cerevisiae*. J Bacteriol. 1971;105:826-830.

204. Kama R, Robinson M, Gerst JE. Btn2, a Hook1 ortholog and potential Batten disease-related protein, mediates late endosome-Golgi protein sorting in yeast. Mol Cell Biol. 2007;27:605-621.

205. Kamer G, Argos P. Primary structural comparison of RNA-dependent polymerase from plant, animal and bacterial viruses. Nucleic Acids Res. 1984;12:7269-7282.

206. Kasschau KD, Carrington JC. A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. Cell. 1998;95:461-470.

207. Kawakami K, Pande S, Faiola B, et al. A rare tRNA-Arg(CCU) that regulates Ty1 element ribosomal frameshifting is essential for Ty1 retrotransposition in *Saccharomyces cerevisiae*. Genetics. 1993;135:309-320.

208. Ke N, Irwin PA, Voytas DF. The pheromone response pathway activates transcription of Ty5 retrotransposons located within silent chromatin. EMBO J. 1997;16:6272-6280.

209. Kelly AC, Shewmaker FP, Kryndushkin D, Wickner RB. Sex, prions and plasmids in yeast. Proc Natl Acad Sci U S A. 2012;109:E2683-E2690.

210. Kennell JC, Moran JV, Perlman PS, Butow RA, Lambowitz AM. Reverse transcriptase activity associated with maturase-encoding group II introns in yeast mitochondria. Cell. 1993;73:133-146.

211. Khemici V, Linder P. RNA helicases in RNA decay. Biochem Soc Trans. 2018;46:163-172.

212. Kicka S, Bonnet C, Sobering AK, Ganesan LP, Silar P. A mitotically inheritable unit containing a MAP kinase module. Proc Natl Acad Sci U S A. 2006;103:13445-13450.

213. Kiktev DA, Melomed MM, Lu CD, Newnam GP, Chernoff YO. Feedback control of prion formation and propagation by the ribosome-associated chaperone complex. Mol Microbiol. 2015;96:621-632.

214. Kim JM, Vanguri S, Boeke JD, Gabriel A, Voytas DF. Transposable elements and genome organization: a comprehensive survey of retrotransposons revealed by the complete *Saccharomyces cerevisiae* genome sequence. Genome Res. 1998;8:464-478.

215. Kimura Y, Takaoka M, Tanaka S, et al. N(alpha)-acetylation and proteolytic activity of the yeast 20S proteasome. J Biol Chem. 2000;275:4635-4639.

216. King C-Y, Diaz-Avalos R. Protein-only transmission of three yeast prion strains. Nature. 2004;428:319-323.

217. King C-Y, Tittmann P, Gross H, Gebert R, Aebi M, Wuthrich K. Prion-inducing domain 2-114 of yeast Sup35 protein transforms in vitro into amyloid-like filaments. Proc Natl Acad Sci U S A. 1997;94:6618-6622.

218. Kinsey JA. Transnuclear retrotransposition of the Tad element of *Neurospora*. Proc Natl Acad Sci U S A. 1993;90:9384-9387.

219. Kinsey PT, Sandmeyer SB. Ty3 transposes in mating populations of yeast. Genetics. 1995;139:81-94.

220. Kirkland PA, Reidy M, Masison DC. Functions of yeast Hsp40 chaperone Sis1p dispensable for prion propagation but important for prion curing and protection from prion toxicity. Genetics. 2011;188:565-577.

221. Kishimoto A, Hasegawa K, Suzuki H, Taguchi H, Namba K, Yoshida M. beta-Helix is a likely core structure of yeast prion Sup35 amyloid fibers. Biochem Biophys Res Commun. 2004;315(3):739-745.

222. Kondo H, Chiba S, Toyoda K, Suzuki N. Evidence for negative-strand RNA virus infection in fungi. Virology. 2013;435:201-209.

223. Koonin EV. Evolution of double-stranded RNA viruses: a case of polyphyletic origin from different groups of positive-stranded RNA viruses. Semin Virol. 1992;3:327-339.

224. Koonin EV, Choi GH, Nuss DL, Shapira R, Carrington JC. Evidence for common ancestry of a chestnut blight hypovirulence-associated double-stranded RNA and a group of positive-strand RNA plant viruses. Proc Natl Acad Sci U S A. 1991;88:10647-10651.

225. Kraus A, Hoyt F, Schwartz CL, et al. High-resolution structure and strain comparison of infectious mammalian prions. Mol Cell. 2021;81(21):4540-4551.e6. doi: 10.1016/j.molcel.2021.08.011

226. Kryndushkin D, Ihrke G, Piermartiri TC, Shewmaker F. A yeast model of optineurin proteinopathy reveals a unique aggregation pattern associated with cellular toxicity. Mol Microbiol. 2012;86:1531-1547.

227. Kryndushkin D, Shewmaker F, Wickner RB. Curing of the [URE3] prion by Btn2p, a Batten disease-related protein. EMBO J. 2008;27:2725-2735.

228. Kryndushkin D, Smirnov VN, Ter-Avanesyan MD, Kushnirov VV. Increased expression of Hsp40 chaperones, transcriptional factors, and ribosomal protein Rpp0 can cure yeast prions. J Biol Chem. 2002;277:23702-23708.

229. Kryndushkin D, Wickner RB. Nucleotide exchange factors for Hsp70s are required for [URE3] prion propagation in *Saccharomyces cerevisiae*. Mol Biol Cell. 2007;18:2149-2154.

230. Kryndushkin DS, Alexandrov IM, Ter-Avanesyan MD, Kushnirov VV. Yeast [*PSI*+] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104. J Biol Chem. 2003;278:49636-49643.

231. Kumar J, Reidy M, Masison DC. Yeast J-protein Sis1 prevents prion toxicity by maintaining prion protein solubility. Genetics. 2021;219(2):iyab129.

232. Kumar N, Gaur D, Gupta A, Puri A, Sharma D. Hsp90-associated immunophilin homolog Cpr7 is required for the mitotic stability of [URE3] prion in *Saccharomyces cerevisiae*. PLoS Genet. 2015;11:e1005567. doi:10.1371/journal.pgen.1005567

233. Kushnirov VV, Kochneva-Pervukhova NV, Cechenova MB, Frolova NS, Ter-Avanesyan MD. Prion properties of the Sup35 protein of yeast *Pichia methanolica*. EMBO J. 2000;19:324-331.

234. Lacroute F. Non-Mendelian mutation allowing ureidosuccinic acid uptake in yeast. J Bacteriol. 1971;106:519-522.

235. Lalucque H, Malagnac F, Brun S, Kicka S, Silar P. A non-Mendelian MAPK-generated hereditary unit controlled by a second MAPK pathway in *Podospora anserina*. Genetics. 2012;191:419-433.

236. Lambowitz AM, Zimmerly S. Mobile group II introns. Annu Rev Genet. 2004;38:1-35.

237. Lambowitz AM, Zimmerly S. Group II introns: mobile ribozymes that invade DNA. Cold Spring Harb Perspect Biol. 2011;3(8):a003616. doi:10.1101/cshperspect.a003616

238. Lancaster DL, Dobson CM, Rachubinski RA. Chaperone proteins select and maintain [PIN+] prion conformations in *Saccharomyces cerevisiae*. J Biol Chem. 2013;288:1266-1276.

239. Lee B-S, Bi L, Garfinkel DJ, Bailis AM. Nucleotide excision repair/TFIIH helicases Rad3 and Ssl2 inhibit short-sequence recombination and Ty1 transposition by similar mechanisms. Mol Cell Biol. 2000;20:2436-2445.

240. Lee Y-J, Wickner RB. *MAK10*, a glucose-repressible gene necessary for replication of a dsRNA virus of *Saccharomyces cerevisiae*, has T cell receptor a-subunit motifs. Genetics. 1992;132:87-96.

241. Leibowitz MJ, Wickner RB. A chromosomal gene required for killer plasmid expression, mating, and spore maturation in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A. 1976;73(6):2061-2065.

242. Lesage P, Todeschini AL. Happy together: the life and times of Ty retrotransposons and their hosts. Cytogenet Genome Res. 2005;110:70-90.

243. Levin HL, Boeke JD. Demonstration of retrotransposition of the Tf1 element in fission yeast. EMBO J. 1992;11:1145-1153.

244. Levin HL, Weaver DC, Boeke JD. Novel gene expression mechanism in a fission yeast retroelement: Tf1 proteins are derived from a single primary translation product. EMBO J. 1993;12:4885-4895.

245. Liebman SW, Newman G. A ubiquitin-conjugating enzyme, *RAD6*, affects the distribution of Ty1 retrotransposon integration positions. Genetics. 1993;133:499-508.

246. Lin J-H, Levin HL. A complex structure in the mRNA of Tf1 is recognized and cleaved to generate the primer of reverse transcription. Genes Dev. 1997;11:270-285.

247. Liu L, Cheng J, Fu Y, Liu H, Jiang D, Xie J. New insights into reovirus evolution: implications from a newly characterized mycoreovirus. J Gen Virol. 2017;98:1132-1141.

248. Liu L, Xie J, Cheng J, et al. Fungal negative-stranded RNA virus that is related to bornaviruses and nyaviruses. Proc Natl Acad Sci U S A. 2014;111:12205-12210.

249. Liu S, Xie J, Cheng J, et al. Fungal DNA virus infects a mycophagous insect and utilizes it as a transmission vector. Proc Natl Acad Sci U S A. 2016;113:12803-12808.

250. Liu Y, Dieckmann CL. Overproduction of yeast virus-like particle coat protein genome in strains deficient in a mitochondrial nuclease. Mol Cell Biol. 1989;9:3323-3331.

251. Lolle S, Skipper N, Bussey H, Thomas DY. The expression of cDNA clones of yeast M1 double-stranded RNA in yeast confers both killer and immunity pheonotypes. EMBO J. 1984;3:1283-1387.

252. Loubradou G, Begueret J, Turcq B. MOD-D, a Galpha subunit of the fungus Podospora anserina, is involved in both regulation of development and vegetative incompatibility. Genetics. 1999;152:519-528.

253. Lund PM, Cox BS. Reversion analysis of [psi-] mutations in *Saccharomyces cerevisiae*. Genet Res. 1981;37:173-182.

254. Maddelein ML, Dos Reis S, Duvezin-Caubet S, Coulary-Salin B, Saupe SJ. Amyloid aggregates of the HET-s prion protein are infectious. Proc Natl Acad Sci U S A. 2002;99(11):7402-7407.

255. Malagon F, Jensen TH. The T-body, a new cytoplasmic RNA granule in *Saccharomyces cerevisiae*. Mol Cell Biol. 2008;28:6022-6032.

256. Malagon F, Jensen TH. T-body formation precedes virus-like particle maturation in *S. cerevisiae*. RNA Biol. 2011;8:184-189.

257. Malinovska L, Kroschwald S, Munder MC, Richter D, Alberti S. Molecular chaperones and stress-inducible protein-sorting factors coordinate the spaciotemporal distribution of protein aggregates. Mol Biol Cell. 2012;23:3041-3056.

258. Margittai M, Langen R. Fibrils with parallel in-register structure constitute a major class of amyloid fibrils: molecular insights from electron paramagnetic resonance spectroscopy. Q Rev Biophys. 2008;41:265-297.

259. Marschalek R, Hofmann J, Schumann G, Gosseringer R, Dingermann T. Structure of DRE, a retrotransposable element which integrates with position specificity upstream of *Dictyostelium discoideum* tRNA genes. Mol Cell Biol. 1992;12:229-239.

260. Martegani E, Vanoni M, Mauri I, Rudoni S, Saliola M, Alberghina L. Identification of gene encoding a putative RNA-helicase, homologous to SKI2, in chromosome VII of Saccharomyces cerevisiae. Yeast. 1997;13(4):391-397.

261. Masison DC, Blanc A, Ribas JC, Carroll K, Sonenberg N, Wickner RB. Decoying the cap- mRNA degradation system by a dsRNA virus and poly(A)-mRNA surveillance by a yeast antiviral system. Mol Cell Biol. 1995;15:2763-2771.

262. Masison DC, Maddelein M-L, Wickner RB. The prion model for [URE3] of yeast: spontaneous generation and requirements for propagation. Proc Natl Acad Sci U S A. 1997;94:12503-12508.

263. Masison DC, Reidy M. Yeast prions are useful for studying protein chaperones and protein quality control. Prion. 2015;9:174-183.

264. Masison DC, Wickner RB. Prion-inducing domain of yeast Ure2p and protease resistance of Ure2p in prion-containing cells. Science. 1995;270:93-95.

265. Mata CP, Rodriguez JM, Suzuki N, Caston JR. Structure and assembly of double-stranded RNA mycoviruses. Adv Virus Res. 2020;108:213-247.

266. Matsuda E, Garfinkel DJ. Posttranslational interference of Ty1 retrotransposition by antisense RNAs. Proc Natl Acad Sci U S A. 2009;106:15657-15662.

267. Matsumoto Y, Fishel R, Wickner RB. Circular single-stranded RNA replicon in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A. 1990;87(19):7628-7632.

268. Matsumoto Y, Wickner RB. Yeast 20 S RNA replicon. Replication intermediates and encoded putative RNA polymerase. J Biol Chem. 1991;266(19):12779-12783.

269. Matsuo Y, Ikeuchi K, Saeki Y, et al. Ubiquitination of stalled ribosome triggers ribosome-associated quality control. Nat Commun. 2017;8:159.

270. Matthews GD, Goodwin TJD, Butler MI, Berryman TA, Poulter RTM. pCal, a highly unusual Ty1/*copia* retrotransposon from the pathogenic yeast *Candida albicans*. J Bacteriol. 1997;179:7118-7128.

271. Matveenko AG, Barbitoff YA, Jay-Garcia LM, Chernoff YO, Zhouravleva GA. Differential effects of chaperones on yeast prions: CURrent views. Curr Genet. 2018;64:317-325.

272. Maxwell PH, Curcio MJ. Host factors that control long terminal repeat retrotransposons in *Saccharomyces cerevisiae*: implications for the regulation of mammalian retroviruses. Eukaryot Cell. 2007;6:1069-1080.

273. McGlinchey R, Kryndushkin D, Wickner RB. Suicidal [PSI+] is a lethal yeast prion. Proc Natl Acad Sci U S A. 2011;108:5337-5341.

274. Meinhardt F, Kast A, Voges R, Schroth M, Schaffrath R, Klassen R. Autoselection of cytoplasmic yeast virus like elements encoding toxin/antitoxin systems involves a nuclear barrier for immunity gene expression. PLoS Genet. 2015;11:e1005005.

275. Mertens PPC, Hillman BI, Suzuki N, et al, eds. VIIIth Report of the International Committee on Taxonomy of Viruses. Elsevier; 2005.

276. Meskauskas A, Baxter JL, Carr EA, et al. Delayed rRNA processing results in significant ribosome biogenesis and functional defects. Mol Cell Biol. 2003;23:1602-1613.

277. Milgroom MG, Cortesi P. Biological control of chestnut blight with hypovirulence: a critical analysis. Annu Rev Phytophathol. 2004;42:311-338.

278. Mochama P, Jadhav P, Neupane A, Marzano S-YL. Mycoviruses as triggers and targets of RNA silencing in white mold fungus *Sclerotinia sclerotiorum*. Viruses. 2018;10:214.

279. Moore SP, Garfinkel DJ. Correct integration of model substrates by Ty1 integrase. J Virol. 2000;74:11522-11530.

280. Moosavi B, Wongwigkam J, Tuite MF. Hsp70/Hsp90 co-chaperones are required for efficient Hsp104-mediated elimination of the yeast [PSI+] prion but not for prion propagation. Yeast. 2010;27:167-179.

281. Mueller MW, Allmaier M, Eskes R, Schweyen RJ. Transposition of group II intron aI1 in yeast and invasion of mitochondrial genes at new locations. Nature. 1993;366:174-176.

282. Nagy PD. Yeast as a model host to explore virus-host interactions. Annu Rev Phytopathol. 2008;46:217-242.

283. Naito T, Kiyasu Y, Sugiyama K, et al. An influenza virus replicon system in yeast identified Tat-SF1 as a stimulatory host factor for viral RNA synthesis. Proc Natl Acad Sci U S A. 2007;104:18235-18240.

284. Naitow H, Canady MA, Wickner RB, Johnson JE. L-A dsRNA virus at 3.4 Angstroms resolution reveals particle architecture and mRNA decapping mechanism Nat Struct Biol. 2002;9:725-728.

285. Nakayashiki T, Kurtzman CP, Edskes HK, Wickner RB. Yeast prions [URE3] and [*PSI*+] are diseases. Proc Natl Acad Sci U S A. 2005;102:10575-10580.

286. Natsoulis G, Thomas W, Roghmann MC, Winston F, Boeke JD. Ty1 tranposition in *Saccharomyces cerevisiae* is nonrandom. Genetics. 1989;123:269-279.

287. Natsuaki T, Natsuaki KT, Okuda S, et al. Relationships between the cryptic and temperate viruses of alfalfa, beet and white clover. Intervirology. 1986;25:69-75.

288. Nelson RJ, Ziegilhoffer T, Nicolet C, Werner-Washburne M, Craig EA. The translation machinery and 70 kDal heat shock protein cooperate in protein synthesis. Cell. 1992;71:97-105.

289. Ness F, Ferreira P, Cox BS, Tuite MF. Guanidine hydrochloride inhibits the generation of prion “seeds” but not prion protein aggregation in yeast. Mol Cell Biol. 2002;22:5593-5605.

290. Nuss DL. Hypovirulence: mycoviruses at the fungal-plant interface. Nat Rev Microbiol. 2005;3:632-642.

291. Oh C-S, Hillman BI. Genome organization of a partitivirus from the filamentous ascomycete *Atkinsonella hypoxylon*. J Gen Virol. 1995;76:1461-1470.

292. Ohtake Y, Wickner RB. Yeast virus propagation depends critically on free 60S ribosomal subunit concentration. Mol Cell Biol. 1995;15:2772-2781.

293. Osaki H, Wei CZ, Arakawa M, et al. Nucleotide sequences of double-stranded RNA segments from a hypovirulent strain of the white root rot fungus *Rosellinia necatrix:* possibility of the first member of the *Reoviridae* from fungus. Virus Genes. 2002;25:101-107.

294. Parent KN, Takagi Y, Cardone G, et al. Structure of a protozoan virus frm the human genitourinary parasite *Trichomonas vaginalis*. MBio. 2013;4:e00056-13.

295. Patel BK, Gavin-Smyth J, Liebman SW. The yeast global transcriptional co-repressor protein Cyc8 can propagate as a prion. Nat Cell Biol. 2009;11:344-349.

296. Patel BK, Liebman SW. "Prion proof" for [PIN+]: infection with in vitro-made amyloid aggregates of Rnq1p-(132-405) induces [PIN+]. J Mol Biol. 2007;365:773-782.

297. Patino MM, Liu J-J, Glover JR, Lindquist S. Support for the prion hypothesis for inheritance of a phenotypic trait in yeast. Science. 1996;273:622-626.

298. Paul CP, Fulbright DW. Double-stranded RNA molecules from Michigan hypovirulent isolates of *Endothia parasitica* vary in size and sequence homology. Phytopathology. 1988;78:751-755.

299. Paushkin SV, Kushnirov VV, Smirnov VN, Ter-Avanesyan MD. Propagation of the yeast prion-like [*psi+*] determinant is mediated by oligomerization of the *SUP35*-encoded polypeptide chain release factor. EMBO J. 1996;15:3127-3134.

300. Paushkin SV, Kushnirov VV, Smirnov VN, Ter-Avanesyan MD. *In vitro* propagation of the prion-like state of yeast Sup35 protein. Science. 1997;277:381-383.

301. Picologlou S, Brown N, Leibman SW. Mutations in *RAD6*, a yeast gene encoding a ubiquitin-conjugating enzyme, stimulate retrotransposition. Mol Cell Biol. 1990;10:1017-1022.

302. Pierce MM, Baxa U, Steven AC, Bax A, Wickner RB. Is the prion domain of soluble Ure2p unstructured? Biochemistry. 2005;44(1):321-328.

303. Plant EP, Dinman JD. Torsional restraint: a new twist on frameshifting pseudoknots. Nucleic Acids Res. 2005;30:1825-1833.

304. Polashock JJ, Bedker PJ, Hillman BI. Movement of a small mitochondrial double-stranded RNA element of *Cryphonectria parasitica*: ascospore inheritance and implications for mitochondrial recombination. Mol Gen Genet. 1997;256:566-571.

305. Polashock JJ, Hillman BI. A small mitochondrial double-stranded (ds) RNA element associated with a hypovirulent strain of the chestnut blight fungus and ancestrally related to yeast cytoplasmic T and W dsRNAs. Proc Natl Acad Sci U S A. 1994;91:8680-8684.

306. Price BD, Rueckert RR, Ahlquist P. Complete replication of an animal virus and maintenance of expressioin vectors derived from it in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A. 1996;93:9465-9470.

307. Puri A, Singh P, Kumar N, Kumar R, Sharma D. Tah1, a key component of R2TP complex that regulates assembly of snoRNP, is involved in de novo generation and maintenance of yeast prion [URE3]. J Mol Biol. 2021;433(13):166976. doi:10.1016/j.jmb.2021.166976

308. Pyle JD, Keelng PJ, Nibert ML. Amalga-like virus infecting *Antonospora locustae*, a microsporidian pathogen of grasshoppers, plus related viruses associated with other arthropods. Virus Res. 2017;233:95-104.

309. Qian Z, Huang H, Hong JY, et al. Yeast Ty1 retrotransposition is stimulated by a synergistic interaction between mutations in chromatin assembly factor I and histone regulatory proteins. Mol Cell Biol. 1998;18:4783-4792.

310. Ramirez-Garrastacho M, Esteban R. Yeast RNA viruses as indicators of exosome activity: human exosome hCsl4p participates in RNA degradation in *Saccharomyces cerevisiae*. Yeast. 2011;28:821-832.

311. Reidy M, Masison DC. Sti1 regulation of Hsp70 and Hsp90 is critical for curing of *Saccharomyces cerevisiae* [PSI+] prions by Hsp104. Mol Cell Biol. 2010;30:3542-3552.

312. Reidy M, Miot M, Masison DC. Prokaryotic chaperones support yeast prions and thermotolerance and define disaggregation machinery interactions. Genetics. 2012;192:185-193.

313. Resende CG, Outeiro TF, Sands L, Lindquist S, Tuite MF. Prion protein gene polymorphisms in *Saccharomyces cerevisiae*. Mol Microbiol. 2003;49:1005-1017.

314. Rhee SK, Icho T, Wickner RB. Structure and nuclear localization signal of the SKI3 antiviral protein of *Saccharomyces cerevisiae*. Yeast. 1989;5(3):149-158.

315. Ribas JC, Fujimura T, Wickner RB. Essential RNA binding and packaging domains of the Gag-Pol fusion protein of the L-A double-stranded RNA virus of *Saccharomyces cerevisiae*. J Biol Chem. 1994;269:28420-28428.

316. Ribas JC, Wickner RB. RNA-dependent RNA polymerase consensus sequence of the L-A double-stranded RNA virus: definition of essential domains. Proc Natl Acad Sci U S A. 1992;89(6):2185-2189.

317. Riek R, Saupe SJ. The HET-S/s prion motif in the control of cell death. Cold Spring Harb Perspect Biol. 2016;8:a023515.

318. Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Seraphin B. A generic protein purification method for protein complex characterization and proteome exploration. Nat Biotechnol. 1999;17:1030-1032.

319. Rigling D, Alfen NKV. Regulation of laccase biosynthesis in the plant pathogenic fungus *Cryphonectria parasitica* by double-stranded RNA. J Bacteriol. 1991;173:8000-8003.

320. Ritter C, Maddelein ML, Siemer AB, et al. Correlation of structural elements and infectivity of the HET-s prion. Nature. 2005;435(7043):844-848.

321. Rizet G. Les phenomenes de barrage chez *Podospora anserina*: analyse genetique des barrages entre les souches s et S. Rev Cytol Biol Veg. 1952;13:51-92.

322. Roberts BT, Wickner RB. A class of prions that propagate via covalent auto-activation. Genes Dev. 2003;17:2083-2087.

323. Robzyk K, Recht J, Osley MA. Rad6-dependent ubiquitination of histone H2B in yeast. Science. 2000;287:501-504.

324. Rockwell NC, Thorner JW. The kindest cuts of all: crystal structures of Kex2 and furin reveal secrets of precursor processing. Trends Biochem Sci. 2004;29:80-87.

325. Rodriguez-Cousino N, Esteban R. Both yeast W double-stranded RNA and its single-stranded form 20S RNA are linear. Nucleic Acids Res. 1992;20:2761-2766.

326. Rodriguez-Cousino N, Solorzano A, Fujimura T, Esteban R. Yeast positive-strand virus-like RNA replicons. 20S and 23S RNA terminal nucleotide sequences and 3′ end secondary structures resemble those of RNA coliphages. J Biol Chem. 1998;273:20363-20371.

327. Rodriguez CN, Esteban LM, Esteban R. Molecular cloning and characterization of W double-stranded RNA, a linear molecule present in *Saccharomyces cerevisiae*. Identification of its single-stranded RNA form as 20 S RNA. J Biol Chem. 1991;266(19):12772-12778.

328. Romanova NV, Chernoff YO. Hsp104 and prion propagation. Protein Pept Lett. 2009;16:598-605.

329. Ross ED, Baxa U, Wickner RB. Scrambled prion domains form prions and amyloid. Mol Cell Biol. 2004;24:7206-7213.

330. Ross ED, Edskes HK, Terry MJ, Wickner RB. Primary sequence independence for prion formation. Proc Natl Acad Sci U S A. 2005;102:12825-12830.

331. Russell PJ, Bennett AM, Love Z, Baggott DM. Cloning, sequencing and expression of a full-length cDNA copy of the M1 double-stranded RNA virus from the yeast, *Saccharomyces cerevisiae*. Yeast. 1997;13:829-836.

332. Sadish H, Rampelt H, Shorter J, et al. Hsp110 chaperones regulate prion formation and propagation in *S. cerevisiae* by two discrete activities. PLoS One. 2008;3:e1763.

333. Safadi RA, Talarek N, Jacques N, Aigle M. Yeast prions: could they be exaptations? The *URE2/*[URE3] system in *Kluyveromyces lactis*. FEMS Yeast Res. 2011;11:151-153.

334. Saha A, Mitchell JA, Nishida Y, et al. Gag restricts Ty1 retrotransposition and mediates copy number control. J Virol. 2015;89:3922-3938.

335. Salinero AC, Knoll ER, Zhu ZI, Landsman D, Curcio MJ, Morse RH. The mediator co-activator complex regulates Ty1 retromobility by controlling the balance between Ty1i and Ty1 promoters. PLoS Genet. 2018;12:e1007232.

336. Santoso A, Chien P, Osherovich LZ, Weissman JS. Molecular basis of a yeast prion species barrier. Cell. 2000;100:277-288.

337. Sasaki A, Onoue M, Kanematsu S, et al. Extending chestnut blight hypovirus host range within diaporthales by biolistic delevery of viral cDNA. Mol Plant Microbe Interact. 2005;15:780-789.

338. Sato Y, Shamsi W, Jamal A, Bhatti MF, Kondo H, Suzuki N. Hadaka Virus 1: a capsidless eleven-segmented positive-sense single-stranded RNA virus from a phytopathogenic fungus, *Fusarium oxysporum*. MBio. 2020;11:e00450-20.

339. Saupe SJ. Molecular genetics of heterokaryon incompatibility in filamentous ascomycetes. Microbiol Mol Biol Rev. 2000;64:489-502.

340. Schlumpberger M, Prusiner SB, Herskowitz I. Induction of distinct [URE3] yeast prion strains. Mol Cell Biol. 2001;21(20):7035-7046.

341. Schmidt C, Kowalinski E, Shanmuganathan V, et al. The cryo-EM structure of a ribosome-Ski2-Ski3-Ski8 helicase complex. Science. 2016;354:1431-1433.

342. Schmitt MJ. Cloning and expression of a cDNA copy of the viral K28 killer toxin gene in yeast. Mol Gen Genet. 1995;246:236-246.

343. Searfoss AM, Wickner RB. 3′ poly(A) is dispensable for translation. Proc Natl Acad Sci U S A. 2000;97:9133-9137.

344. Segers GC, van Wezel R, Zhang X, Hong Y, Nuss DL. Hypovirus papain-like protease p29 suppresses RNA silencing in the natural fungal host and in a heterologous plant system. Eukaryot Cell. 2006;5:896-904.

345. Segers GC, Zhang X, Deng F, Sun Q, Nuss DL. Evidence that RNA silencing functions as an antiviral defense mechanism in fungi. Proc Natl Acad Sci U S A. 2007;104:12902-12906.

346. Seol JH, Shevchenko A, Shevchenko A, Deshales RJ. Skip1 forms multiple protein complexes, including RAVE, a regulator of V-ATPase assembly. Nat Cell Biol. 2001;3:384-391.

347. Shapira R, Choi GH, Nuss DL. Virus-like genetic organization and expression strategy for a double-stranded RNA genetic element associated with biological control of chestnut blight. EMBO J. 1991;10:731-739.

348. Shapira R, Nuss DL. Gene expression by a hypovirulence-associated virus of the chestnut blight fungus involves two papain-like protease activities. J Biol Chem. 1991;266:19419-19425.

349. Sharma D, Masison DC. Hsp70 structure, function, regulation and influence on yeast prions. Protein Pept Lett. 2009;16:571-581.

350. Shen Y, Bruenn JA. RNA structural requirements for RNA binding, replication, and packaging in the yeast double-stranded RNA virus. Virology. 1993;195:481-491.

351. Shewmaker F, Mull L, Nakayashiki T, Masison DC, Wickner RB. Ure2p function is enhanced by its prion domain in *Saccharomyces cerevisiae*. Genetics. 2007;176:1557-1565.

352. Shewmaker F, Wickner RB, Tycko R. Amyloid of the prion domain of Sup35p has an in-register parallel b-sheet structure. Proc Natl Acad Sci U S A. 2006;103:19754-19759.

353. Shigemoto K, Brennan J, Walls E, et al. Identification and characterization of a developmentally regulated mammalian gene that utilizes -1 ribosomal frameshifting. Nucleic Acids Res. 2001;29:4079-4088.

354. Shorter J. The mammalian disaggregase machinery: Hsp110 synergizes with Hsp70 and Hsp40 to catalyze protein disaggregation and reactivation in a cell-free system. PLoS One. 2011;6:e26319.

355. Shorter J, Lindquist S. Prions as adaptive conduits of memory and inheritance. Nat Rev Genet. 2005;6:435-450.

356. Singh AC, Helms C, Sherman F. Mutation of the non-Mendelian suppressor, Psi, in yeast by hypertonic media. Proc Natl Acad Sci U S A. 1979;76:1952-1956.

357. Sommer SS, Wickner RB. Gene disruption indicates that the only essential function of the SKI8 chromosomal gene is to protect Saccharomyces cerevisiae from viral cytopathology. Virology. 1987;157(1):252-256.

358. Son M, Choi H, Kim KH. Specific binding of *Fusarium graminearum* Hex1 protein to untranslated regions of the genomic RNA of Fusarium graminearum virus 1 correlates with increased accumulation of both strands of virus RNA. Virology. 2016;489:202-211.

359. Son M, Lee KM, Yu J, et al. The HEX1 gene of Fusarium graminearum is required for fungal asexual reproduction and pathogenesis and for efficient viral RNA accumulation of Fusarium graminearum virus 1. J Virol. 2013;87:10356-10367.

360. Son M, Wickner RB. Nonsense-mediated mRNA decay factors cure most [PSI+] prion variants. Proc Natl Acad Sci U S A. 2018;115: E1184-E1193. doi:10.1073/pnas.1717495115

361. Son M, Wickner RB. Normal levels of ribosome-associated chaperones cure two groups of [PSI+] variants. Proc Natl Acad Sci U S A. 2020;117:26298-26306.

362. Sondheimer N, Lindquist S. Rnq1: an epigenetic modifier of protein function in yeast. Mol Cell. 2000;5:163-172.

363. Sondheimer N, Lopez N, Craig EA, Lindquist S. The role of Sis1 in the maintenance of the [RNQ+] prion. EMBO J. 2001;20:2435-2442.

364. Specht S, Miller SBM, Mogk A, Bukau B. Hsp42 is required for sequestration of protein aggregates into deposition sites in *Saccharomyces cerevisiae*. J Cell Biol. 2011;195:617-629.

365. Speransky V, Taylor KL, Edskes HK, Wickner RB, Steven A. Prion filament networks in [URE3] cells of *Saccharomyces cerevisiae*. J Cell Biol. 2001;153:1327-1335.

366. Stahl G, Ben Salem S, McCarty G, Raman A, Shah M, Farabaugh PJ. Programmed +1 translational frameshifting in the yeast *Saccharomyces cerevisiae* results from the disruption of translational error correction. Cold Spring Harb Symp Quant Biol. 2001;66:249-258.

367. Stauder CM, Nuss DL, Zhang DX, et al. Enhanced hypovirus transmission by engineered super donor strains of the chestnut blight fungus, *Cryphonectria parasitica*, into a natural population of strains exhibiting diverse vegetative compatibility genotypes. Virology. 2019;528:1-6.

368. Steidle EA, Chong LS, Wu M, et al. A novel inositol pyrophosphate phosphatase in *Saccharomyces cerevisiae*: Siw14 protein selectively cleaves the b-phosphate from 5-diphosphoinositol pentakisphosphate (5PP-IP5). J Biol Chem. 2016;291:6772-6783.

369. Sun Q, Choi GH, Nuss DL. A single Argonaute gene is required for induction of RNA silencing antiviral defense and promotes viral recombination. Proc Natl Acad Sci U S A. 2009;106:17927-17932.

370. Sundararajan A, Lee BS, Garfinkel DJ. The Rad27 (Fen-1) nuclease inhibits Ty1 mobility in *Saccharomyces cerevisiae*. Genetics. 2003;163:55-67.

371. Suzuki G, Shimazu N, Tanaka M. A yeast prion, Mod5, promotes acquired drug resistance and cell survival under environmental stress. Science. 2012;336:355-359.

372. Suzuki N, Supyani S, Maruyama K, Hillman BI. Complete genome sequence of Mycoreovirus-1/Cp9B21, a member of a novel genus within the family Reoviridae, isolated from the chestnut blight fungus, *Cryphonectra parasitica*. J Gen Virol. 2004;85:3437-3448.

373. Tanaka M, Chien P, Naber N, Cooke R, Weissman JS. Conformational variations in an infectious protein determine prion strain differences. Nature. 2004;428:323-328.

374. Tanaka M, Collins SR, Toyama BH, Weissman JS. The physical basis of how prion conformations determine strain phenotypes. Nature. 2006;442:585-589.

375. Tang J, Naitow H, Gardner NA, et al. The structural basis of recognition and removal of cellular mRNA 7-methyl G 'caps' by a viral capsid protein: a unique viral response to hose defense. J Mol Recognit. 2005;18:158-168.

376. Taylor DJ, Bruenn J. The evolution of novel fungal genes from nonretroviral RNA viruses. BMC Biol. 2009;7:88.

377. Taylor KL, Cheng N, Williams RW, Steven AC, Wickner RB. Prion domain initiation of amyloid formation *in vitro* from native Ure2p. Science. 1999;283:1339-1343.

378. Ter-Avanesyan MD, Dagkesamanskaya AR, Kushnirov VV, Smirnov VN. The *SUP35* omnipotent suppressor gene is involved in the maintenance of the non-Mendelian determinant [psi+] in the yeast *Saccharomyces cerevisiae*. Genetics. 1994;137:671-676.

379. Tercero JC, Dinman JD, Wickner RB. Yeast *MAK3* N-acetyltransferase recognizes the N-terminal four amino acids of the major coat protein (*gag*) of the L-A double-stranded RNA virus. J Bacteriol. 1993;175:3192-3194.

380. Tercero JC, Riles LE, Wickner RB. Localized mutagenesis and evidence for post-transcriptional regulation of MAK3, a putative N-acetyltransferase required for dsRNA virus propagation in *Saccharomyces cerevisiae*. J Biol Chem. 1992;267:20270-20276.

381. Tercero JC, Wickner RB. MAK3 encodes an N-acetyltransferase whose modification of the L-A *gag* N-terminus is necessary for virus particle assembly. J Biol Chem. 1992;267:20277-20281.

382. Tessarz P, Mogk A, Bukau B. Substrate threading through the central pore of the Hsp104 chaperone as a common mechanism for protein disaggregation and prion propagation. Mol Microbiol. 2008;68:87-97.

383. Toh-e A, Guerry P, Wickner RB. Chromosomal superkiller mutants of Saccharomyces cerevisiae. J Bacteriol. 1978;136(3):1002-1007.

384. Troisi EM, Rockman ME, Nguyen PP, Oliver EE, Hines JK. Swa2, the yeast homolog of mammalian auxilin, is specifically required for the propagation of the prion variant [URE3-1]. Mol Microbiol. 2015;97:926-941.

385. Tucker JM, Larango ME, Wachsmuth LP, Kannan N, Garfinkel DJ. The Ty1 retrotransposon restriction protein p22 targets Gag. PLoS Genet. 2015;11:e1005571.

386. Tuite MF, Mundy CR, Cox BS. Agents that cause a high frequency of genetic change from [*psi*+] to [*psi*-] in *Saccharomyces cerevisiae*. Genetics. 1981;98:691-711.

387. Turcq B, Deleu C, Denayrolles M, Begueret J. Two allelic genes responsible for vegetative incompatibility in the fungus *Podospora anserina* are not essential for cell viability. Mol Gen Genet. 1991;288:265-269.

388. Tycko R. Physical and structural basis for polymorphism in amyloid fibrils. Protein Sci. 2014;23:1528-1539.

389. Umland TC, Taylor KL, Rhee S, Wickner RB, Davies DR. The crystal structure of the nitrogen catabolite regulatory fragment of the yeast prion protein Ure2p. Proc Natl Acad Sci U S A. 2001;98:1459-1464.

390. Vainio EJ, Chiba S, Ghabrial SA, et al. ICTV virus taxonomy profile: *Partitiviridae*. J Gen Virol. 2018;99:17-18.

391. Van Arsdell SW, Stetler GL, Thorner J. The yeast repeated element sigma contains a hormone-inducible promoter. Mol Cell Biol. 1987;7:749-759.

392. Vimaladithan A, Farabaugh PJ. Special peptidyl-tRNA molecules can promote translational frameshifiting without slippage. Mol Cell Biol. 1994;14:8107-8116.

393. Voytas DF, Boeke JD. Ty1 and Ty5 of *Saccharomyces cerevisiae*. In: Craig NL, Gellert CRM, Lambowitz AL, eds. Mobile DNA II. ASM Press; 2002:631-662.

394. Wang L, He H, Wang S, et al. Evidence for a novel negative-stranded RNA mycovirus isolated from the plant pathogenic fungus *Fusarium graminearum*. Virology. 2018;518:232-240.

395. Wasmer C, Lange A, Van Melckebeke H, Siemer AB, Riek R, Meier BH. Amyloid fibrils of the HET-s(218-279) prion form a beta solenoid with a triangular hydrophobic core. Science. 2008;319:1523-1526.

396. Wesolowski M, Wickner RB. Two new double-stranded RNA molecules showing non-mendelian inheritance and heat inducibility in *Saccharomyces cerevisiae*. Mol Cell Biol. 1984;4(1):181-187.

397. Wickner RB. [URE3] as an altered *URE2* protein: evidence for a prion analog in *S. cerevisiae*. Science. 1994;264(5158):566-569.

398. Wickner RB, Beszonov E, Bateman DA. Normal levels of the antiprion proteins Btn2 and Cur1 cure most newly formed [URE3] prion variants. Proc Natl Acad Sci U S A. 2014;111:E2711-E2720.

399. Wickner RB, Dyda F, Tycko R. Amyloid of Rnq1p, the basis of the [*PIN*+] prion, has a parallel in-register b-sheet structure. Proc Natl Acad Sci U S A. 2008;105:2403-2408.

400. Wickner RB, Edskes HE. Yeast killer elements hold their hosts hostage. PLoS Genet. 2015;11:e1005139.

401. Wickner RB, Edskes HE, Son M, Wu S, Niznikiewicz M. Innate immunity to prions: anti-prion systems turn a tsunami of prions into a slow drip. Curr Genet. 2021;67:833-847.

402. Wickner RB, Edskes HK, Kryndushkin DS, McGlinchey R, Bateman D, Kelly A. Prion diseases of yeast: amyloid structure and biology. Semin Cell Dev Biol. 2011;22:469-475.

403. Wickner RB, Kelly AC, Bezsonov EE, Edskes HE. Prion propagation is controlled by inositol polyphosphates. Proc Natl Acad Sci U S A. 2017;114:E8402-E8410.

404. Wickner RB, Shewmaker F, Bateman DA, et al. Yeast prions: structure, biology and prion-handling systems. Microbiol Mol Biol Rev. 2015;79:1-17.

405. Wickner RB, Shewmaker F, Kryndushkin D, Edskes HK. Protein inheritance (prions) based on parallel in-register b-sheet amyloid structures. Bioessays. 2008;30:955-964.

406. Widner WR, Matsumoto Y, Wickner RB. Is 20S RNA naked? Mol Cell Biol. 1991;11(5):2905-2908.

407. Widner WR, Wickner RB. Evidence that the *SKI* antiviral system of *Saccharomyces cerevisiae* acts by blocking expression of viral mRNA. Mol Cell Biol. 1993;13:4331-4341.

408. Wilhelm M, Wilhelm F-X. Cooperation between reverse transcriptase and integrase during reverse transcription and formation of the preintegrative complex of Ty1. Eukaryot Cell. 2006;5:1760-1769.

409. Wilke CM, Adams J. Fitness effects of Ty transposition in *Saccharomyces cerevisiae*. Genetics. 1992;131:31-42.

410. Williamson VM, Cox D, Young ET, Russell DW, Smith M. Characterization of transposable element-associated mutations that alter yeast alcohol dehydrogenase II expression. Mol Cell Biol. 1983;3:20-31.

411. Winkler J, Tyedmers J, Bukau B, Mogk A. Chaperone networks in protein disaggregation and prion propagation. J Struct Biol. 2012;179:152-160.

412. Winkler J, Tyedmers J, Bukau B, Mogk A. Hsp70 targets Hsp100 chaperones to substrates for protein disaggregation and prion fragmentation. J Cell Biol. 2012;198:387-404.

413. Winston F. Analysis of *SPT* genes: a genetic approach toward analysis of TFIID, histones, and other transcription factors of yeast. In: McKnight SL, Yamamoto KR, eds. Transcription Regulation. Vol 2. Cold Spring Harbor Laboratory Press; 1992:1271-1293.

414. Winston F, Chaleff DT, Valent B, Fink GR. Mutations affecting Ty-mediated expression of the *HIS4* gene in *Saccharomyces cerevisiae*. Genetics. 1984;107:179-197.

415. Wu S, Cheng J, Fu Y, et al. Virus-mediated suppression of host non-self recognition facilitates horizontal transmission of heterologous viruses. PLoS Pathog. 2017;13:e1006234.

416. Xie W, Gai X, Zhu Y, Zappulla DC, Sternglanz R, Voytas DF. Targeting of the yeast Ty5 retrotransposon to silent chromatin is mediated by interactions between integrase and Sir4p. Mol Cell Biol. 2001;21:6606-6614.

417. Xu H, Boeke JD. Host genes that influence transposition in yeast: the abundance of a rare tRNA regulates Ty1 transposition frequency. Proc Natl Acad Sci U S A. 1990;87:8360-8364.

418. Xu H, Boeke JD. Localization of sequences required in *cis* for yeast Ty1 element transposition near the long terminal repeats: analysis of mini-Ty elements. Mol Cell Biol. 1990;10:2695-2702.

419. Xu H, Boeke JD. Inhibition of Ty1 transposition by mating pheromones in *Saccharomyces cerevisiae*. Mol Cell Biol. 1991;11:2736-2743.

420. Yieh L, Hatzis H, Kassavetis G, Sandmeyer SB. The Brf and TATA-binding protein subunits of the RNA polymerase III transcription factor IIIB mediate position-specific integration of the gypsy - like element Ty3. J Biol Chem. 2000;275:29800-29807.

421. Yu X, Li B, Fu Y, et al. A geminivirus-related DNA mycovirus that confers hypovirulence to a plant pathogenic fungus. Proc Natl Acad Sci U S A. 2010;107:8387-8392.

422. Yu X, Li B, Fu Y, et al. Extracellular transmission of a DNA mycovirus and its use as a natural fungicide. Proc Natl Acad Sci U S A. 2013;110:1452-1457.

423. Yuan AH, Hochschild A. A bacterial global regulator forms a prion. Science. 2017;355:198-201.

424. Zhang DX, Nuss DL. Engineering super mycovirus donor strains of chestnut blight fungus by systematic disruption of multilocus *vic* genes. Proc Natl Acad Sci U S A. 2016;113:2062-2067.

425. Zhang R, Hisano S, Tani A, Kondo H, Kanematsu S, Suzuki N. A capsidless ssRNA virus hosted by an unrelated dsRNA virus. Nat Microbiol. 2016;1:15001. doi:10.1038/nmicrobiol.2015.1

426. Zhang X, Nuss DL. A host dicer is required for defective viral RNA production and recombinant virus vector instability for a positive sense RNA virus. Proc Natl Acad Sci U S A. 2008;105:16749-16754.

427. Zhang X, Segers GC, Sun Q, Deng F, Nuss DL. Characterization of hypovirus-derived small RNAs generated in the chestnut blight fungus by an inducible DCL-2-dependent pathway. J Virol. 2008;82:2613-2619.

428. Zhang Y, Valentín Gesé G, Conz C, et al. The ribosome-associated complex RAC serves in a relay that directs nascent chains to Ssb. Nat Commun. 2020;11:1504.

429. Zinoviev A, Ayupov RK, Abaeva IS, Hellen CUT, Pestova TV. Extraction of mRNA from stalled ribosomes by the Ski complex. Mol Cell. 2020;77:1340-1349.

430. Zou S, Voytas DF. Silent chromatin determines target preference of the *Saccharomyces* retrotransposon Ty5. Proc Natl Acad Sci U S A. 1997;94:7412-7416.

CHAPTER 21

Bacteriophages

Graham F. Hatfull

Brief history

***Caudovirales*: dsDNA tailed phages**

Bacteriophage λ and the "Lambdoid" phages

Bacteriophage T4

Other tailed phages

Bacteriophage HK97

Bacteriophage T7

Bacteriophage P22

Bacteriophages P2 and P4

Bacteriophage G

Bacteriophage Mu

Bacteriophages P1 and N15

Bacteriophage ø29

Insights from other phage families

Tectiviridae: dsDNA, linear, with proteins on 5′ ends

Microviridae: ssDNA, circular

Inoviridae: ssDNA, filamentous

Leviviridae: +ssRNA

Cystoviridae: dsRNA, segmented

Corticoviridae: dsDNA, circular

Plasmaviridae: circular dsDNA

Evolution of phages

Genome mosaicism

Common ancestry of archaeal and eukaryal viruses with phages

Phage parts in other contexts

Pyocins

Gene transfer agents

Encapsulins

Type VI secretion systems

Host interactions

Parasitic interactions

DNA replication

Antagonistic interactions

Symbiotic interactions

Phage communities and ecology

Prospects for therapeutic uses of phages

Acknowledgments

Bacteriophages—viruses that infect bacteria—collectively constitute a majority of biological entities on planet Earth. Virions of the tailed double-stranded DNA (dsDNA) phages, which are the most easily identified in environmental samples, are present at roughly 10^7/mL in coastal seawater and at comparably high numbers in numerous other environmental situations examined.[200] A rough calculation indicates that there are about 10^{31} individual tailed phage virions on the planet, a literally astronomical number, which means that if all the tailed phages were laid end to end, they would extend for 200 million light years into intergalactic space.[89,150] These numbers become even more impressive when we consider that most bacterial genomes contain from one to two dozen phage genomes as prophages.[36] Measurements of the persistence of phages in the environment suggest that the entire population of 10^{31} individual virions turns over every 4-5 days,[219] and this leads to the estimate that it takes roughly 10^{24} productive infections per second to maintain the population—speaking only of the tailed phages.[89] It should be no surprise that bacteriophages were incredibly diverse and that their genomes have complex evolutionary histories dominated by pervasive horizontal genetic exchange and genomic mosaicism.[85]

Brief History

Bacteriophages were discovered in 1915 and 1917, based on their ability to cause clearing (cell lysis) in bacterial lawns and to be propagated as infectious agents.[55,205] The first ~20 years of research on phages was aimed at understanding the basis of these phenomena, and in the process these studies contributed to developing ideas about the nature of viruses more generally. There was also keen interest during this time in phage therapy, the idea of using phages as agents to combat bacterial infections in human patients.[197] The "modern" era of phage research began in about 1940 when a diverse group of physicists and biologists who shared an interest in understanding the nature of the gene began to use phages as their experimental model system in this quest. This approach was wildly successful, playing a dominant role in the development of the field of molecular biology and leading over a period of ~35 years to much of our current understanding of not only the nature of the gene but also the ways that the information in genes is expressed and how that expression is regulated. Along the way, work on phages also made fundamental contributions to the field of virology. Phage research during this time was concentrated on a small number of phages, with the result that we now have a very deep understanding of a handful of individual phages. A crowning achievement of this era of phage-dominated molecular biology was the development in the early 1970s of the methods of recombinant DNA. This made many of the overwhelming technical advantages of phages as experimental systems available for other organisms, including eukaryotes, and the center of gravity of molecular biology research shifted away from phages, though important basic research on phage life cycles has continued to the present. In recent years, new themes have come to the fore in phage research that relate to natural phage populations and their effects on the environment and to phage evolution, including the influence of the phage population and its evolution on the evolution of their host bacteria.[57] These studies are inspired and made rational by the numbers cited in the first paragraph of this chapter, and they are made possible by the development of high-throughput sequencing technology and by the availability of the detailed knowledge of the biology of phages developed over the past 75 years.[85] Studies on the assembly and structure of phage virions have had central roles in structural virology and have given insights into biological structure and assembly more generally.[102] Finally, throughout the history of phage biology there has been interest in the application of phages and their components to practical problems, and this has continued to the present, including but certainly not limited to a renewed interest in phage therapy.[86]

This chapter will first consider the main features of the life cycles of the different groups of phages that have been identified, essentially summarizing the detailed studies of individual "model" phages with an emphasis on what general principles of viral lifestyle are illustrated by each. For some of these, particularly the dsDNA tailed phages, there are enough examples of different phages in the group that we can begin to identify which features of the life cycles are universal among the group and which are either "optional" or accomplished by different mechanisms by different phages. We will then consider questions about phage populations and phage evolution—questions that are informed by the very large numbers cited in the first paragraph of this chapter.

The International Committee on the Taxonomy of Viruses (ICTV) has made multiple changes to bacteriophage taxonomies over the past few years, with the inclusion of many new families and some restructuring of prior groups.[5] Perhaps the most highly represented taxonomic group in nature are the dsDNA tailed phages in the order *Caudovirales*, which has been expanded to include many additional families (*Autographiviridae*, *Chaseviridae*, *Demerecviridae*, *Drexlerviridae*, *Finnlakeviridae*, *Halspiviridae*, *Herelleviridae*, *Ovaliviridae*, and *Thaspiviridae*) in addition to its traditional *Myoviridae*, *Siphoviridae*, and *Podoviridae* families[4] (Table 21.1). These traditional families are based on their tail morphologies (Table 21.1). Seven additional families encompass groups of viruses with common ancestries, and shared features including nucleic acid chemistries (Table 21.1). Prototypes of each have been defined (Table 21.1) and will be described in further detail below. Nonetheless, the tailed phages (*Caudovirales*) are special in the sense that they are likely the numerically most abundant group of viruses on the planet and almost certainly the most genetically diverse. They are also the most extensively studied group of phages and so will occupy most of the space in what follows.

TABLE 21.1 Some ICTV-designated phage families and their prototypes

Family	Prototypes	Characteristics
Siphoviridae	λ	dsDNA, Long noncontractile tails
Myoviridae	T4	dsDNA, contractile tails
Podoviridae	T7	dsDNA, short, stubby tails
Tectiviridae	PRD1	dsDNA, linear 5′ proteins, internal membrane
Microviridae	ϕX174	ssDNA, circular, icosahedral
Inoviridae	M13	ssDNA, filamentous
Leviviridae	MS2, Qβ	ssRNA. small icosahedral
Cystoviridae	ϕ6	dsRNA, segmented; enveloped
Corticoviridae	PM2	dsDNA, circular; internal membrane
Plasmaviridae	L2	dsDNA, circular; enveloped

dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

CAUDOVIRALES: dsDNA TAILED PHAGES

The dsDNA tailed phages are tremendously diverse genetically, to the extent that a majority of the protein sequences encoded in a newly determined phage genome sequence typically fail to make any matches in a database search, other than to closely related relatives.[38,85] Genome sizes of known tailed phages range from a low of <15 kilobase pairs (kbp) up to around 500 kbp, a range of more than 30-fold.[90] As will become evident, they are also very diverse in the specifics of their life cycles, including how they interact with the host cell. On the other hand, the similarities these viruses share across this range of diversity are so extensive as to leave no doubt that they share (distant) common ancestry, at least for the genetic features like the capsid protein genes that they all have.[94]

The head of a tailed phage consists of a protein shell based on icosahedral symmetry that is usually a rather straightforward manifestation of the principles of capsid structure described by Caspar and Klug.[43] There is no lipid membrane. Replicated dsDNA is pumped into the empty protein shell through a 12-subunit grommetlike structure at one vertex of the icosahedral shell, known as the *portal*, or sometimes the *connector*. DNA is packaged in these viruses to an unusually high density, roughly the same as the density in a DNA crystal.[116] Following DNA packaging, the portal, together with some adaptor proteins, serves as the attachment point for the tail.[168] The morphology of the tail is quite variable among different tailed phages, but it always has the same functions, namely, to attach to the surface of the host cell and serve as a conduit for delivering the DNA into the cytoplasm of the cell. The bulk of the virion protein is left on the outside of the cell after DNA injection, a circumstance that made possible the classical Hershey/Chase experiment[95] that showed that the genetic material is DNA and not protein. Once the DNA arrives in the cytoplasm, the genetic diversity among different phages alluded to earlier is manifested as diversity in the details of their life cycles, and specific examples of the life cycles of a few well-studied phages are given later. At a fundamental level, these phages can be divided into "virulent" and "temperate" categories. Virulent phages always enter the lytic life cycle, in which infection always leads to production of progeny virions and their release by cell lysis, with cell death. Temperate phages, in contrast, make a "choice" soon after infection between lytic growth and entering the lysogenic cycle, in which gene expression from the phage DNA is largely repressed and the phage becomes a part of the genetic complement of the host cell—now called a *prophage*—and replicates as part of the cellular DNA without killing the cell for an indefinite number of generations.

In the following sections, we consider in moderate detail the life cycles of two very well-studied tailed phages, λ and T4, both phages of *Escherichia coli* and some of the most extensively studied viruses. These two have served as important model experimental systems since the 1940s and 1950s. They were the initial source of much of our understanding about the nature of tailed phages, and their numerous differences highlight the diversity seen among the well-studied tailed phages. There are numerous other tailed phages that have made similarly important contributions to our understanding, and we have chosen several of them to illustrate features of their life cycles that are not represented in the two phages that are discussed in detail. Some of the topics considered here have been the subjects of intensive study by multiple investigators for 50 years or more, and in such cases, it may not be possible to cite all the relevant primary literature; if so, useful reviews will be cited that give access to the primary literature.

Bacteriophage λ and the "Lambdoid" Phages

The K12 strain of *E coli* was isolated from a pediatric patient at Stanford University Hospital in 1922. Some 30 years later, in the course of bacterial genetics experiments with K12, Lederberg and Lederberg[121] discovered a temperate phage that was carried as a prophage in the K12 genome, which they named λ. Phage λ subsequently became the most extensively studied of a small number of temperate phages that were chosen for experimental investigation. Other phages that are similar to λ in gene organization and lifestyle, and which can form recombinants with λ at reasonably high frequency, have been termed *lambdoid* phages.[32] Some of the lambdoid phages have been studied in their own right, and all of them and their hybrids with λ have had an important role in illuminating some of the most fundamental aspects of the temperate phage lifestyle.[91]

Phage λ's dsDNA genome is 48 503 bp in length (Fig. 21.1).[92,185] The DNA molecule in the virion has its ends at discrete sequences, and there are 12 base single-stranded 5′ extensions at the ends. The extensions are complementary in sequence, allowing the linear molecule to circularize by base pairing between these "cohesive ends," which it does immediately upon being injected into the host cell. The two joined ends are then covalently joined by the host enzyme, DNA ligase.[222]

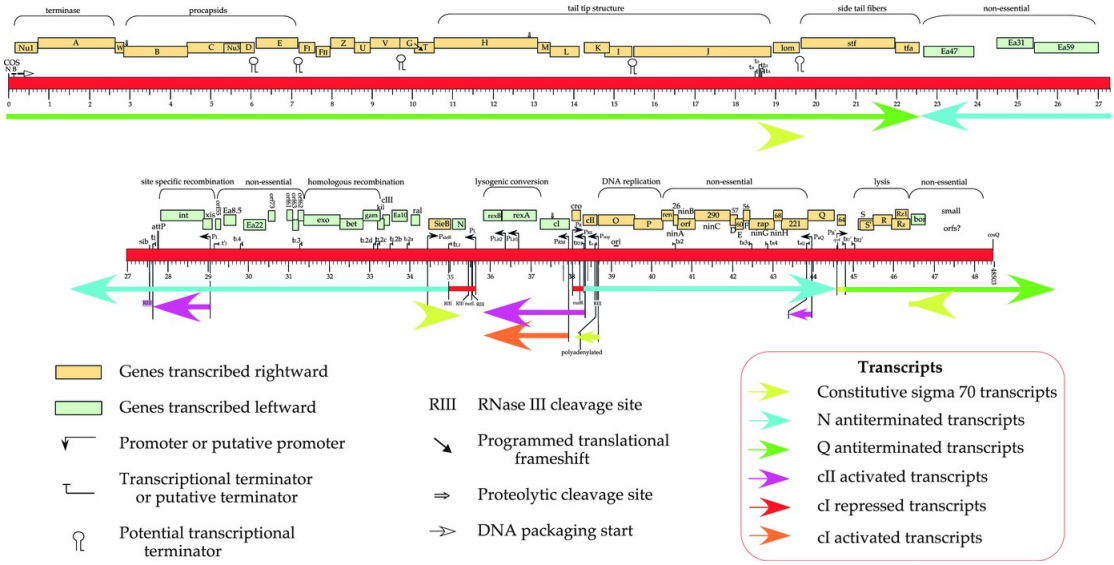

FIGURE 21.1 **Map of the phage λ genome**. λ DNA is represented as the *red bar* with genes shown as boxes above; functions are indicated.

The λ virion has an isometric head (capsid) with T = 7 icosahedral symmetry and a long noncontractile tail (Fig. 21.2). It infects its *E coli* host by binding to the surface of the cell first through its long "side tail fibers," which bind to the outer membrane protein OmpC, and then through an interaction between the host outer membrane protein, LamB, and the phage protein, gpJ, located at the tip of the tail.[214] Only this second interaction (gpJ:LamB) is essential for successful infection; the side tail fibers, which make adsorption kinetics faster, were lost through a mutation early in the laboratory history of λ.[92] (Note on nomenclature: in the phage literature, gpX refers to the protein encoded by gene *X*, with "gp" meaning "gene product" and *not* "glycoprotein.") Details of the DNA injection process are not well understood but are thought to include a rearrangement of some of the proteins that make up the tail tip to facilitate passage across the cell envelope, a process for which there is experimental support in some other phages but not yet in λ. One thing that must happen before the DNA can pass out of the head, down the tail tube, and into the cell is that the tail length tape measure protein, gpH, a few copies of which occupy much of the lumen of the tail, must exit. There is biochemical evidence that gpH is injected into membrane vesicles if the membranes contain the LamB receptor protein,[179] and there is genetic evidence that gpH must interact with an inner membrane sugar transport protein, PtsM, in order for injection to be accomplished successfully.[178]

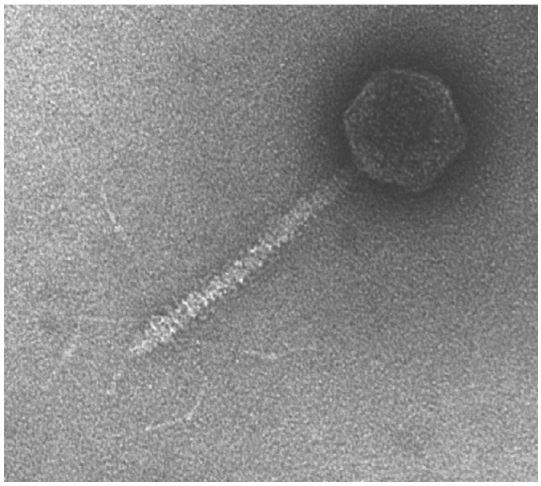

FIGURE 21.2 **The bacteriophage λ virion**. Electron micrograph of λ, negatively stained with uranyl acetate. Scale: the length of the tail, excluding fibers, is ~150 nm.

(Courtesy of Robert Duda.)

Following DNA injection and circularization, λ makes a decision between the lytic and lysogenic life cycles. We first describe the lytic cycle, in which the phage programs the cell to produce a crop of progeny virions and releases them by cell lysis at the end of the growth cycle. Following that, we describe the lysogenic cycle, in which the expression of most phage genes is repressed and the phage DNA integrates into the continuity of the host chromosome as a prophage.

The Lytic Cycle

All transcription of λ genes is carried out by the host RNA polymerase, unmodified for immediate early transcription and altered in its properties by interaction with phage-encoded proteins for subsequent temporal classes of transcripts.[78] The genes are generally arranged in large operons, making it possible to regulate their transcription by regulating a small number of promoters. Upon DNA entry, RNA polymerase initiates transcription at the two divergent immediate early promoters, P_L and P_R, completing transcription of just one gene in each case, gene *N* and gene *cro*, respectively, before encountering a ρ-dependent terminator and stopping.[153] The N protein, gpN, is a transcription antitermination factor that renders polymerase insensitive to termination signals by forming a complex with polymerase, several host factors, and a specific signal near the start of the nascent transcript called *nut*, for Nutilization. The RNA polymerase, altered in this fashion, now reads through the terminators and into early genes farther down the P_L and P_R controlled operons. The most relevant of these genes for the lytic cycle are the *O* and *P* genes, which mediate phage-specific DNA replication, and the *Q* gene, which has a central role in mediating late gene transcription, all transcribed from the P_R promoter, and three genes for recombination enzymes, transcribed from P_L.[53] An important feature of the *nut* sites is that they only act *in cis*—that is, gpN can cause antitermination only for a polymerase that starts from a promoter with a *nut* site near the beginning of the transcript, which in λ means only P_L and P_R. A consequence of this is that some transcribing polymerases can be insensitive to termination signals at the same time that others are susceptible to termination, depending on which promoter they started at. This differential sensitivity to termination signals figures in the regulation of the proteins responsible for integration and excision of the prophage, described later.

Transcription of the late genes starts about 10 minutes after the start of infection, from the P_R' promoter located downstream from the *Q* gene (Fig. 21.1). The P_R' promoter is a strong promoter, recognized by the unmodified host RNA polymerase, but the polymerase requires the action of the early phage protein gpQ to read through a strong pause site located soon after the promoter.[176] As with polymerase that has been acted on by gpN, polymerase acted on by gpQ is insensitive to termination signals and reads through the entire ~26-kb late gene operon, which includes the lysis genes, head genes, and tail and tail fiber genes. Despite the fact that all of the late genes are transcribed equally, the molar amounts of the proteins produced by translation of the messenger RNA (mRNA) vary more than 1000-fold due to differences in efficiency of translation initiation.[40] The amounts of the proteins produced appear to be appropriate to what is required for efficient assembly of virions.

Phage DNA replication is carried out by the host replication machinery, but that machinery is directed to the phage replication origin by two phage-encoded early proteins, gpO and gpP. gpO binds to the origin, four tandem imperfect 19-bp repeats within the gene *O* coding region, to form a compact complex to which gpP binds.[213] gpP recruits the host replication helicase, DnaB, to the complex; the rest of the host replication machinery joins; and gpO and gpP are removed by the host chaperones, DnaK, DnaJ, and GrpE, which allows the replication complex to start replication.[164] Replication is initially bidirectional, producing more unit-length circles by a "theta" mode of replication, but at 10-12 minutes after infection it shifts to a unidirectional rolling circle mode to produce the multigenome concatemer that is the DNA substrate for packaging into heads.

The heads and tails of phage λ are assembled separately and join to form virions after their assembly is complete.[88] Purified heads and tails will join efficiently *in vitro* to make infectious virions, implying that no additional factors are required for this step of assembly. Tail assembly starts with a complex of the proteins that will form the tip (head-distal) end of the tail. The initial complex is joined by a few copies of the *tape measure protein*, gpH, which extends the length of the tail as an α-helix and provides a template around which the major tail protein, gpV, polymerizes as 32 stacked rings of 6 subunits each.[106] This polymerization is thought to be mediated by two proteins, gpG and gpGT, which are essential for assembly but absent from the mature tail, and which are therefore regarded as tail assembly chaperones. The two chaperones are produced as the result of an inefficient translational frameshift between two overlapping open reading frames, an arrangement that is highly conserved among other long-tailed phages.[223] Following assembly of the tail tube, two adaptor proteins add to the top of the tail and it is ready to join to a head.

As with all other characterized tailed phages, λ head assembly first produces an empty protein shell—the prohead or procapsid—into which DNA is packaged. Four phage-encoded proteins participate in prohead assembly; the major capsid protein, gpE; the portal protein, gpB; the protease, gpC; and the scaffolding protein, gpNu3.[44] The details of how these proteins come together to assemble accurately into a T = 7 capsid are not entirely clear, but the assembly is thought to be nucleated by the portal, a 12-subunit grommetlike structure that occupies one vertex of the otherwise icosahedrally symmetric prohead.[168] Assembly of the major capsid (coat) protein requires the participation of similar numbers of the self-assembling scaffolding protein.[139] How the scaffolding protein contributes to this process remains the central mystery of how capsid assembly is achieved. In the absence of scaffolding protein, the capsid protein assembles into "monsters," sheets, tubes, and misshapen spirals of capsid protein that have apparently lost the ability to insert pentamer vertices into the hexagonal lattice of capsid proteins with sufficient accuracy and regularity to make an isometric, icosahedrally symmetric structure.

Roughly 10 copies of the protease are assembled into the prohead, and these are responsible for cutting the scaffolding protein into fragments that are lost from the structure. The portal subunit is also proteolytically processed, with 22 amino acids removed from the N-terminus, but unlike what is observed in some phages, the major capsid protein is not processed. The protease itself participates in a curious "protein fusion" reaction, in which a portion of each protease molecule is joined covalently to a copy of the major capsid protein.[138] Following, or concomitant with, the proteolytic maturation of the prohead, DNA packaging commences. DNA is pumped into the procapsid through the ring of portal protein subunits by a phage-encoded, adenosine triphosphate (ATP) cleavage–powered DNA translocase similar to that of phages T4 and ø29 (see later). This molecular motor also contains the nuclease that cleaves *cos* sites to release the packaged DNA from the concatemer that is the substrate for packaging.

Progeny λ particles are released from the cell through a tightly controlled lysis system that ensures the concluding act of lytic growth occurs at the optimal time.[211] λ lysis requires the products of four genes, *S, R, Rz*, and *Rz1*. gpR is the endolysin and cleaves the peptidoglycan layer in the cell wall, but it has no effect on the integrity of the cell until it has access to its substrate, which is prevented by the cytoplasmic membrane. Access, and the timing of lysis, is determined by the products of gene *S*, the 105-residue holin (S105), and the 107-residue antiholin that is translated from an initiation site two codons upstream of that used for S105. The ratio of S105 and S107 is critical for the timing of lysis, which occurs when S105 produces one or more large-sized holes in the membrane.[63] Rz and Rz1 form a *spannin* complex that spans the periplasm and leads to fusion of the inner and outer membranes to ensure efficient and complete lysis.[19,110]

Lysogenic Cycle

The lysogenic cycle starts the same way as the lytic cycle described earlier, with DNA injection and circularization, transcription from P_L and P_R, extension of that transcription by the action of the gpN antitermination protein, and the consequent expression of the early proteins. Among these early proteins are CII ("C-two"), transcribed from P_R, and CIII ("C-three"), transcribed from P_L.[53]

The CII protein plays the central role in the "decision" between the lytic and lysogenic cycles. CII is a transcription factor that binds to and activates the leftward-pointing promoter P_{RE} (*promoter for repressor establishment*). The resulting transcription from P_{RE} goes backward through the *cro* gene and then forward through the leftward-oriented *cI* gene, causing production of the λ repressor, the CI (*C-one*) protein. When it is produced in sufficient quantity, the CI repressor shuts off further transcription from P_L and P_R by binding to the overlapping operators, O_L and O_R, respectively, and occluding the promoters from RNA polymerase binding. In this way, repression is established and can persist for an indefinite number of bacterial generations.

The choice between the lytic and lysogenic cycles is determined by the level of CII protein in the infected cell—with enough CII, transcription from P_{RE} is activated sufficiently to produce enough CI repressor to establish repression, while with low levels of CII, too little CI repressor is made to stop transcription from P_L and P_R, and the lytic cycle goes forward as described earlier. The level of CII protein is determined in turn by environmental factors, principally the physiologic state of the infected cell. The readout of the state of the cell is the level of a cellular protease, FtsH, which degrades CII, and is modulated by HflKC and cyclic adenosine monophosphate (cAMP).[107] In this sense, CII is the sensor with which the phage assesses the state of the cell and decides between the lytic and lysogenic cycles. Another phage protein, CIII, biases the decision in favor of lysogeny by antagonizing the FtsH protease and disfavoring degradation of CII protein.[109] Finally, a high multiplicity of infection favors lysogeny; this is thought to be because a larger number of genomes in the cell means higher production of CII and CIII proteins. More recently, it has been shown that λ preferentially binds to the cell poles—especially at the low multiplicities of infection favoring lytic growth—where FtsH is also localized, suggesting there is a topologic component to the lytic/lysogenic decision.[69]

Once lysogeny is established, expression of the CI repressor maintains the prophage state (Fig. 21.3). CI binds to two tripartite operator sites, O_L and O_R, to repress the early lytic promoters, P_L and P_R, respectively.[125] However, CI also plays an important role in regulating its own expression, acting as an activator of the promoter for repressor maintenance (P_{RM}) when bound to the O_{R1} and O_{R2} sites in O_R, and as a repressor when concentrations are sufficiently high to permit binding to the weaker site, O_{R3}. An important component to repression is the formation of DNA loops formed by cooperative association of repressor tetramers bound at O_{L1} and O_{L2}, and at O_{R1} and O_{R2}; this looping also stimulates activation of P_{RM}.[8] Additional cooperative interactions between O_{R3} and O_{L3} enhance the repression of P_{RM} at high repressor concentrations.[125]

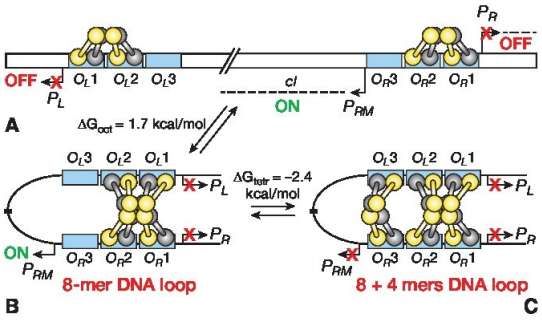

FIGURE 21.3 **DNA looping by the λ repressor**. Models of CI regulation by DNA looping. Detailed conformations of the structures are not known, and maps are not drawn to scale. **A**. Promoters (P_R, P_L, and P_{RM}); operators O_L (O_L1, O_L2, O_L3) and O_R (O_R1, O_R2, O_R3) are in *blue rectangles*; CI dimers (one monomer is shown in *yellow*, the other in *gray*). The bent arrows show the transcription start points of promoters. The dashed line indicates transcripts from P_R, P_L, and P_{RM}. The cI gene is transcribed from P_{RM}. **B**. DNA looping and octamer formation (8-mer) by CI tetramer binding to $O_L1 \sim O_L2$ interacts with that at $O_R1 \sim O_R2$. **C**. Octamer and tetramer (12-mer) of CI binding to O_L and O_R. Red X means promoter is turned off. (Reprinted with permission from Lewis D, Le P, Zurla C, et al. Multilevel autoregulation of λ repressor protein CI by DNA looping in vitro. *Proc Natl Acad Sci U S A*. 2011;108(36):14807-14812.)

The DNA replication genes of a λ prophage are repressed by the action of the CI repressor protein, and the prophage relies on the host replication functions for maintenance in the population as the cell grows and divides. To take advantage of these replication services, the prophage DNA must be integrated into (becomes part of) the host chromosome where it is replicated along with all the other host genes (Fig. 21.4). Integration is catalyzed by the phage-encoded integrase protein (Int), which carries out site-specific, reciprocal recombination between a site on the phage DNA, *attP* (attachment site, phage), and a site on the bacterial chromosome, *attB* (attachment site, bacterium). Because the phage DNA is at this point circular, having joined its cohesive ends immediately upon injection, the Int-mediated reciprocal recombination results in the insertion of a linear form of the phage DNA into the bacterial chromosome. Because the *attP* site, where the phage DNA is broken upon integration, is distant from the *cos* (cohesive ends) site, where the virion DNA was joined, the order of genes in the integrated prophage is a circular permutation of the gene order in the virion DNA. This mechanism of prophage integration, generally known as the *Campbell Model*, seems rather straightforward now; when it was proposed in 1962 by Allan Campbell (the author of this chapter in a previous edition), it was a major conceptual breakthrough.[31,33]

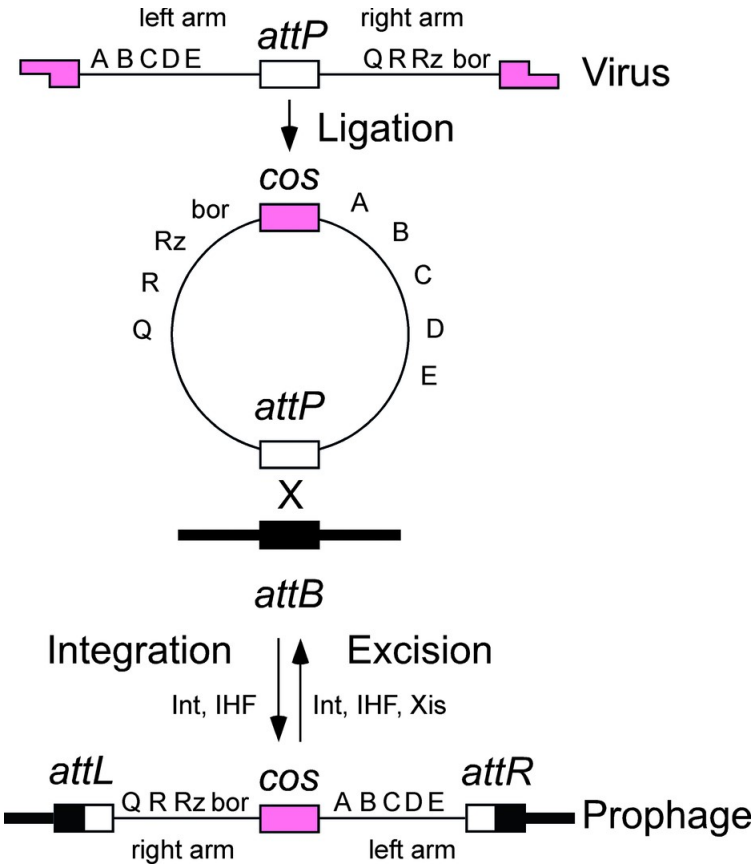

FIGURE 21.4 **Insertion of λ DNA into the bacterial chromosome**. λ virions contain linear DNA with short single-stranded cohesive ends that are ligated to circularize the genome following injection into the cell. *attP* is located near the center of the genome, and the order of some of the genes (A, B, C, etc.) is shown. A prophage is formed by site-specific recombination between the phage attachment site (*attP*) and the bacteria attachment site (*attB*), which is catalyzed by the phage-encoded integrase but also requires integration host factor (IHF). Prophage excision occurs only in the presence of the phage-encoded Xis, which redirects integrase to recombine the attachment junctions *attL* and *attR* to re-create *attP* and *attB*. The virion structure and assembly genes are typically represented as forming the left arm (between *cos* and *attP*) in the viral genome but are permuted relative to the right arm (between *attP* and *cos*) in the prophage.

The *attP* site has a 15-bp "core" sequence that is identical to the corresponding core sequence in *attB*, and it is within the core sequence that the strand exchanges of recombination take place.[117] *AttP* extends on both sides of the core sequence and requires ~240 bp in total. These flanking sequences contain binding sites for Int; for integration host factor (IHF), a host-encoded DNA binding/bending protein; and for Xis and Fis (see later). The *attB* site is only 30 bp long, having fewer such binding sites flanking its core sequence.[117] Integrative recombination takes place within a synaptic complex formed when an *attP*-intasome—containing Int and IHF—captures *attB* DNA.[174] The reaction proceeds with cleavage of the "top" strands of the two attachment sites by Int and reattaching them to the cut strand of the opposite *att* site to form a Holliday junction.[117] The energy of the phosphodiester bond is preserved by two successive transesterifications, first to the active site tyrosine of the integrase and then to the recipient DNA strand, and no additional chemical energy is needed to drive the reaction. Following the top-strand exchange, the complex branch migrates by 7 bp, and bottom-strand exchange takes place by the same mechanism. The reaction product is an integrated prophage flanked by two hybrid attachment sites, *attL* and *attR*.[118]

The lysogenic "cycle" is completed when, after an indefinite number of generations as a repressed prophage, the prophage enters the lytic cycle by the process known as induction. The proximate cause of induction is loss of repression. Induction occurs in about one cell in 10^4-10^6 in a culture of lysogenic cells under laboratory conditions (*spontaneous induction*), but it occurs in nearly 100% of the cells in the culture when they are activated by a moderate dose of ultraviolet light or similar DNA damaging agent. Such treatment causes high production of the cell's RecA protein, which when bound to single-stranded DNA (ssDNA) causes the CI repressor to undergo an autoproteolytic cleavage. This cleavage separates the protein's dimerization domain from its operator-binding domain,[54] and as monomers, the CI operator-binding domains do not have a high enough affinity for their operators to block transcription from P_L and P_R, and so the lytic cycle begins.

An essential part of λ prophage induction is the detachment of the prophage from the host chromosome, termed *excision*. Macroscopically, excision is the reverse of integration—that is, the *attL* and *attR* sites at the ends of the prophage undergo reciprocal recombination to release the circular phage DNA molecule. Excisive recombination requires two protein factors in addition to the Int and IHF needed for integration, namely, the host DNA binding/bending protein Fis and the phage-coded Xis (pronounced "excise") protein. Together, these four proteins and the *attL* and *attR* sites form the excision complex.

Both integration and excision require the catalytic action of integrase, but whether the reaction goes in the direction of integration or excision depends on the absence or presence of Xis (Fig. 21.4). Accordingly, the amount of Xis is differentially regulated by a mechanism that senses whether or not the phage DNA is integrated; if integrated, both Int and Xis are made, allowing excision, and if not, only Int is made, allowing integration. The crucial regulatory site is a sequence named *sib*, located downstream from and across *attP* from the *int* and *xis* genes.[135] *Sib* has the effect of destabilizing mRNA transcribed from P_L (antiterminated by gpN), which includes the sequences of both *xis* and *int*, but it does not

destabilize mRNA from P_I, a CII-dependent promoter within the *xis* coding sequence that encodes only Int. Thus, when *sib* is downstream from *xis* and *int*—that is, when the phage DNA is not integrated—only Int protein can be made and so (provided sufficient CII is made to activate P_I and the phage is headed into the lysogenic cycle) integration ensues. In the integrated prophage, on the other hand, *sib* is no longer downstream from *xis* and *int*, as a result of the integrative recombination. When the prophage is induced, the transcript from P_L, encoding both Xis and Int, is not destabilized, so both proteins are made and excision occurs. Perhaps surprisingly, this elegant and efficient mechanism has been seen only in λ and a few of its closest relatives; there appears to be a great variety of different pathways for achieving the appropriate regulation of prophage integration and excision that have evolved in different temperate phages.

Bacteriophage T4

Bacteriophage T4 is one of seven "type" phages isolated by Delbrück in the 1930s and 1940s that he declared warranted further study.[30] It has received considerable attention from that time to the current day and continues to be a source of new insights into virology, molecular biology, and structural biology.[104] It is virulent and reproduces quickly (<30 minutes), and particles with prolate (ie, elongated) heads and contractile tails contain a dsDNA genome of ~170 kb (Fig. 21.5). Many phages with similar features to T4 have been identified, including two other Delbrück "type" phages, T2 and T6, and phages of other *Proteobacteria* and of the *Cyanobacteria*. These contain genomes ranging from 160 to 250 kbp and range from those with close DNA similarity to T4 to those much more distantly related. Although these relatives contain a "core genome" of about 12%-15% of T4 that are typical of this group, they are otherwise mosaic with common segments interspersed with unrelated genes.[161] Although we will focus here on T4 as the most intensively studied of these, many of the other T4-like phages are likely to share much of its biology.

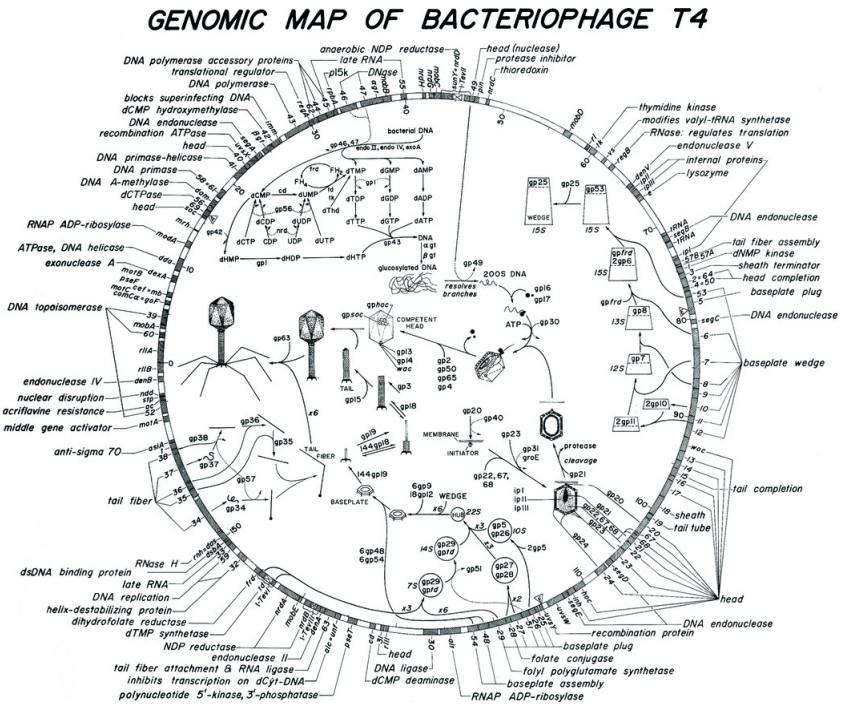

FIGURE 21.5 **Genomic map of phage T4**. The T4 genetic map is circular rather than linear because of the circular permutation of T4 phage chromosomes due to its headful packaging from long concatemers. The general structure of the map was established by standard genetic techniques using classical markers such as those affecting plaque morphology, as well as *amber* mutations that were used to identify many genes. This map shows many of the known and all of the essential genes, based on that classical mapping work and on genome sequence data; the small numbers just inside the circle show the coordinates of the genomic map in kilobase pairs. The names and functions of many genes and the grouping of genes into clusters of related function are shown on the periphery. The branched assembly map in the center shows how an efficient complex of enzymes is produced to make the deoxynucleotides and feed them directly into the T4 DNA polymerase complex (*upper left region*) as well as the branched virion assembly pathway, illustrating how components made in the various branches of the pathway merge to produce an infectious bacteriophage. The small numbers just inside the circle show the coordinates of the genomic map in kilobase pairs. This version of the T4 assembly genomic map is a reduction of a 3-foot-wide hand-drawn map representing an icon in bacteriophage biology. (Adapted by permission from Springer: O'Farrell PH, Kutter E, Nakanishi M. A restriction map of the bacteriophage T4 genome. *Mol Gen Genet*. 1980;179(2):421-435. Copyright © 1980 Springer-Verlag.)

T4 Genome

The T4 genome contains a unique sequence of 169 903 bp, and virion genomes are circularly permuted with terminal redundancies of ~5000 bp[143] (Fig. 21.5). It codes for 160 proteins with known functions, as well as about 120 open reading frames with unknown functions, and several small RNAs, including 8 transfer RNAs (tRNAs). Some parts of the genome are predicted to be mobile, and there are at least 15 homing endonucleases, 3 of which are within introns.[70] T4 DNA is somewhat unusual in that it contains glucosylated hydroxymethylcytosine (hmC), which protects it from host restriction systems and distinguishes it from host DNA that can be degraded and utilized for phage replication.

Expression and Regulation

Expression of T4 genes follows an orderly series of temporally controlled patterns, all involving the use of the host RNA polymerase.[96] However,

a complex series of both covalent and noncovalent modifications to RNA polymerase are required to successfully orchestrate this transcriptional ballet. Transcription of the first T4 genes involves recognition of promoters resembling strong host promoters by $\sigma^{(70)}$-associated host RNA polymerase, but two phage-encoded early products make important modifications to the RNA polymerase. Alt (a protein that is injected with the DNA into the infected cell) catalyzes adenosine diphosphate ribosylation of one α subunit of RNA polymerase, improving the activity of certain T4 early promoters. Two other T4 proteins, ModA and ModB, also adenosine diphosphate-ribosylate RNA polymerase or proteins used in translation during early stages of infection. Another protein, Alc, leads RNA polymerase to selectively terminate transcription prematurely on the host dC-containing DNA while ignoring the viral hmC DNA.[96] As a consequence of these events, transcription of T4 genes readily outcompetes that of the host, with a strong burst of activity within the first minute of infection. The functions of many of these early genes are not known, but two early products, MotA and AsiA, play important roles in the transition from early to middle transcription. Many early T4 mRNAs also disappear quickly at the end of the early phase, largely through the action of RegB, an endoribonuclease that targets early gene transcripts.

MotA is a transcriptional activator that functions along with a co-activator, AsiA, to redirect transcription toward T4 middle promoters. The middle period genes primarily encode functions for DNA metabolism and DNA replication, including *denA* and *denB*, that encode the functions for degrading dC-containing host DNA. About 60 middle promoters have been identified, which contain a host-like −10 sequence (Fig. 21.6) and a MotA box at position −30. MotA binds somewhat weakly to its DNA site but associates strongly with $\sigma^{(70)}$. The co-activator AsiA plays an important role in the transition by also binding to the $\sigma^{(70)}$ subunit of RNA polymerase and conformationally altering the helix-turn-helix DNA binding motif within region 4. As a consequence, recognition of promoter −35 sequences is interrupted (an "antisigma" activity), reducing transcription from both host and T4 early promoters. Thus, MotA and AsiA collaborate to effectively direct the host $\sigma^{(70)}$ to initiate transcription at the unique promoters preceding T4 middle genes.

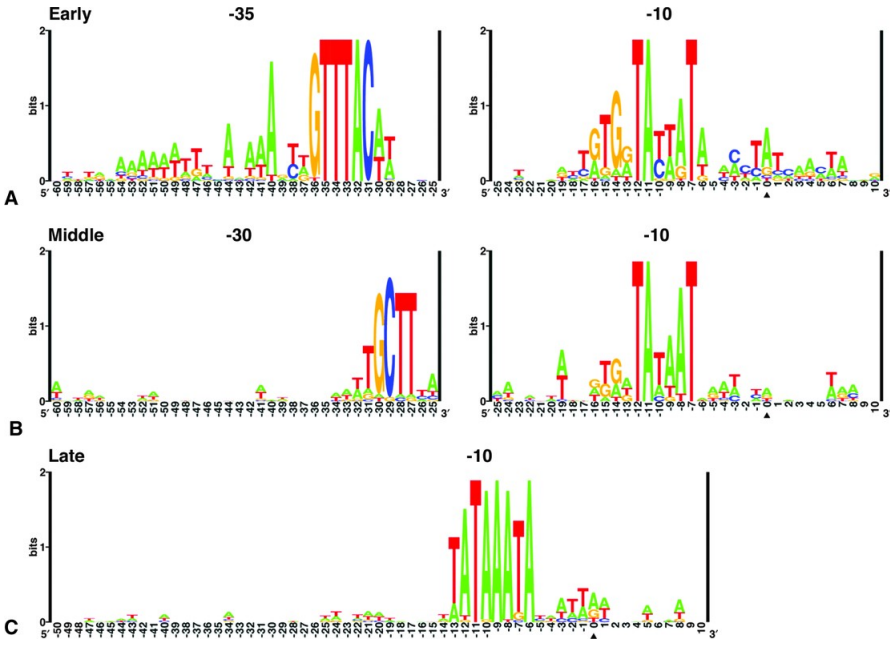

FIGURE 21.6 **Logo of T4 promoters**. Nearly all the sequences in each alignment have promoter activity, as demonstrated by primer extension, transcription from cloned DNA fragments, or RNA hybridization assays. Those promoters included whose start sites have not been mapped all precede a corresponding early, middle, or late gene and show significant similarity to the relevant promoter class. Sequences were independently aligned in the −10, −30, or −35 regions. The information content (Rs) is calculated in "bits" and is the sum of the Rs for each region (except for the late logo, which was calculated from the single alignment at −10). Alignments, logos, and Rs values were obtained as described elsewhere (143) and E. Miller, T. Dean, and T. Schneider, unpublished data). The triangle marks the +1 transcription start site. **A**. Thirty-nine early promoters, Rs = 38.3 bits. **B**. Thirty middle promoters, Rs = 21.1 bits. **C**. Fifty late promoters, Rs = 16.2 bits. (Used with permission of American Society for Microbiology from Miller ES, Kutter E, Mosig G, et al. Bacteriophage T4 genome. *Microbiol Mol Biol Rev*. 2003;67(1):86-156. permission conveyed through Copyright Clearance Center, Inc.)

The transition from early to middle gene expression is also mediated by extension of early T4 transcripts, as many middle genes lie immediately downstream of early genes (Fig. 21.6). Termination of the early transcripts likely occurs at Rho-dependent sites; however, readthrough or antitermination at these sites can give rise to some middle gene expression. However, the antitermination mechanism is unclear. The process could involve an as yet unidentified antiterminator factor (equivalent to λ N or Q; see earlier) or could simply be the consequence of transcription and translational coupling, such as seen when closely linked genes in bacterial operons overcome genetic polarity.[96]

The transition from middle to late T4 gene expression requires the products of genes *33* and *55*.[77] T4 gp55 is a highly divergent member of the $\sigma^{(70)}$ group of proteins and associates with host RNA polymerase to redirect it toward late promoters, which are characterized by an eight–base pair TATA box (5′-TATAAATA-3′) at the position where the −10 site is usually located (Fig. 21.6). There are about 50 related sites in the T4 genome, and many are used to direct late transcription. T4 gp33 binds to the flap tip of the RNA polymerase β subunit, which usually interacts with the region 4 of $\sigma^{(70)}$ such that gp33 mimics this association. T4 gp33 does not recognize a specific DNA sequence (there is no −35 region in T4 late promoters), but it may function by diminishing nonspecific DNA interaction, thus increasing the specific affinity of gp55–RNA polymerase for T4 late promoters.[77]

Interestingly, there is a close linkage between T4 late transcription and DNA replication. T4 gp45, the sliding clamp processivity factor for DNA replication, is strongly implicated in late transcription. It forms "split rings" that load onto DNA with a particular polarity (depending on how the DNA is nicked) and track along the DNA. T4 gp45 and the late transcription factor gp55 associate such that the sliding clamp facilitates the

search for promoters. gp33 is also required for replication-activated transcription at T4 late promoters.

Studies on T4 gene expression also contributed many seminal findings in the area of posttranscriptional control. These include the identification or discovery of translational stop codons, discovery of suppressor tRNAs, tRNA cleavage and subsequent repair by polynucleotide kinase and RNA ligase, protein-mediated translational repression, and group I autocatalytic introns ("ribozymes"). Details of these processes in T4 and T4-related phages are reviewed in Edgell et al.[70] and Uzan and Miller.[207]

Genome Replication

Soon after T4 infection, synthesis of DenA and DenB targets host DNA for degradation to mononucleotides, followed by subsequent conversion of deoxycytidine monophosphate to deoxyhydroxymethylcytosine monophosphate. This is incorporated into T4 DNA during its replication, and the deoxyhydroxymethyl cytosines are subsequently modified by glucosyltransferases. A T4-encoded deoxycytidine-triphosphatase keeps deoxycytidine-triphosphate levels low, so that deoxycytidine-triphosphate does not get errantly incorporated into the newly synthesized T4 genomes. DNA replication occurs in two distinct modes, one that is origin dependent and one that is recombination dependent.

T4 DNA replication is bidirectional and initiates at as many as seven major origins of replication (*oriAi* through *oriG*) early in infection.[112] Initiation requires assembly of a replisome containing several T4 proteins (DNA polymerase, sliding clamp loader, sliding clamp, helicase, primase, and SSB) and synthesis of primers by the host RNA polymerase (to form R-loops). RNA primers can arise from the MotA-dependent promoters at the *ori* sites, and for the lagging strand, from the activity of the T4 primase, gp61. These primers are processed by a phage-encoded RNase H. Coordinated leading- and lagging-strand synthesis then proceeds through to the ends of the viral DNA, generating 3′ extensions (because of the inability to prime synthesis at the extreme 5′ ends of the genomes). Subsequent reprogramming of the host RNA polymerase specificity (see earlier) results in the cessation of this early mode of DNA replication, and a late-expressed protein, UvsW, actively unwinds the RNA-DNA hybrids used for initiation.[67]

The second, late mode of T4 DNA replication is characterized by the dominant role played by homologous recombination and is known as recombination-dependent replication (RDR). The genome ends generated by early (origin-dependent) replication are good substrates and can engage in recombinational pairing with other (co-infecting) genomes or with the other end of the same genome because of the terminal redundancy. Genetic studies suggest that there are multiple pathways by which RDR can proceed, but the primary mode is the formation of D-loops by invasion of an ssDNA end into a DNA duplex, followed by either semiconservative synthesis or synthesis with branch migration.[112] Several T4 proteins are involved in RDR, including UvsX (a RecA-like protein), gp32 (ssDNA binding protein), UvsY (which loads UvsX onto gp32-coated DNA), UvsW (a helicase involved in strand invasion and branch migration), gp59 (for loading the helicase), and the replisome complex. The structures of several of these components have been described.[148] Overall, multiple pathways involving several different phage-encoded enzymes ensure effective replication of the genome. These pathways do not function entirely independently of one another, and thus the T4 system has allowed detailed study of genome replication, recombination, and repair that is germane to both viral and cellular processes.

DNA Packaging

The products of T4 replication are branched, concatemeric networks of DNA in which individual molecules are not distinguishable.[167] Packaging of the DNA into capsids is initiated by the binding of the terminase small subunit protein gp16 to the viral DNA. The small terminase subunit binds as rings (probably 11-mers), and then the gp17 large terminase subunit associates with the protein-DNA complex. This complex associates with the 12-fold symmetric portal protein that sits at a position of one of the capsid pentamers (see later). This "packasome" also contains T4 gp49, a Holliday junction resolvase that removes DNA branches that are otherwise detrimental to packaging. gp17 has two important catalytic domains involved in DNA packaging: an N-terminal adenosine triphosphatase (ATPase) domain and a C-terminal endonuclease domain. Both functions are important for the translocation "motor" activity of the packaging system.

An attractive model for the packaging mechanism is the portal rotation model.[87] The symmetry mismatch between the 5-fold capsid and 12-fold portal means that only one subunit of each can align in any given register. Activation of the terminase ATPase activity could then lead to rotation of the portal and translocation of the DNA, as if a bolt were turning within a nut.[167] Although attractive, single-molecule experiments with the ø29 packaging system (see later) argue against a portal rotation model,[100] other models include a terminase-driven packaging system in which cycles of ATP hydrolysis leads to DNA translocation.[198] Regardless of the mechanistic details, the T4 packaging motor is remarkably powerful when scaled to a macro level, being twice as powerful as a typical automobile engine.[167]

Once packaging begins, the process continues until the capsid is full of densely packed dsDNA (~500 mg/mL)—typically about 103% of the unique genome content—at which point the nuclease activity of the large terminase subunit is triggered to cleave the DNA. Although the terminase-DNA complex dissociates from the filled capsid-portal complex, it may associate with another empty capsid to begin packaging of the DNA from the previous cleavage event. This *headful packaging* process can then continue for as many unit genome lengths present in the concatemer and is the basis for the circularly permuted, terminal redundancy found at the ends of the packaged genome in each virion.

Virion Structure and Assembly

The lunar-lander–shaped T4 virion is one of the most widely recognized icons of molecular biology, and its complex shape requires a correspondingly complex assembly strategy that uses premade subassemblies. These are capsids, tails, and tail fibers that are each made *via* independent pathways that do not merge until the subassemblies are joined together to make a complete virion after DNA packaging is complete. The general outlines of T4 assembly were the result of decades of genetic, biochemical, and structural work. Our understanding of the structure and assembly of each of the subassemblies of the T4 virion has fortunately been enhanced in recent years through structural approaches, primarily cryoelectron microscopy (cryo-EM) and x-ray crystallography.[75,123]

The mature T4 capsid is composed of 960 copies of the cleaved major capsid subunit, gp23*, and 55 copies of the cleaved capsid vertex protein, gp24*, both of which have the HK97-fold. The angular head is a T = 13, Q = 21 elongated icosahedron 86 nm wide by 120 nm long[167] and also contains about 1000 molecules of proteolytically processed internal proteins. The capsid is made initially as a prohead composed of uncleaved proteins that are proteolytically processed only after all prohead components have been added, and after which the proteolytically processed proteins are denoted with a "*" (gp23 is converted to gp23*). Prohead assembly occurs at or around a scaffold core containing gp21 (protease), gp22, gp67, gp68 (scaffolding proteins), gpIPI, gpIPII, gpIPIII, and gpalt (internal proteins) that forms atop a cell membrane–bound portal

complex made of gp20, which later is the site of DNA packaging and tail addition. The prohead grows away from the membrane by simultaneous addition of capsid and core proteins (or in an alternative view, by addition of capsid proteins gp23 and gp24 around a preformed core) until assembly is halted by the formation of the distal cap when the correct length is reached. Assembly is also dependent on the host GroEL chaperone and the phage co-chaperonin T4 gp31 because their action is required for correct folding of the major capsid protein gp23. DNA packaging (see earlier) is accompanied by expansion of the prohead from its initial rounded shape into the angular prolate icosahedron form of the mature capsid. The DNA-filled head is completed by addition of specific proteins to the portal vertex and to the capsid surface. Proteins gp2 and gp4 are apparently added at the portal vertex, followed by neck proteins gp13 and gp14. T4 gp2 is thought to bind to the genome termini and protect the DNA from degradation by the host RecBCD nuclease after injection. Two *decoration proteins*, Hoc and Soc (155 and 870 copies, respectively), are also added to the outside of the shell. Hoc is located at the center of each hexameric gp23 capsomer, and six copies of Soc surround each capsomer, except for the vertices containing gp24. Both Hoc and Soc are dispensable, although Soc does confer additional stability to the capsid. Attached to the neck are also six trimeric whiskers of the Wac protein (also called fibritin), formed by extended coiled-coil structures.

A small set of T4 internal proteins (internal protein I [IPI], IPII, IPIII, and Alt) are specifically localized to the inside of the capsid where they are proteolytically cleaved at specific peptide bonds and subsequently injected into the host along with the DNA. These proteins contain a capsid targeting sequence that can be fused to other proteins such as GFP so as to target their introduction into the capsid.[149] The functions of some internal proteins are known; for example, IPI* protects the incoming DNA from an *E coli* nuclease that is capable of digesting glucosylated hmC DNA (not all *E coli* strains contain this activity, however).

Structure and assembly of the T4 tail is substantially complex, not only because of the larger number of protein components but also because of the dynamic nature of the contractile tail[122] (Fig. 21.7). As with the capsid, a combination of cryo-EM (Fig. 21.8) and crystallography has proven extremely informative in recent years, building on a wealth of genetic and biochemical experiments.[9,228] The tail is composed of the baseplate, a tail sheath surrounding a tail tube, and long tail fibers; altogether it contains about 430 individual polypeptides, and tail assembly requires a total of 22 genes.

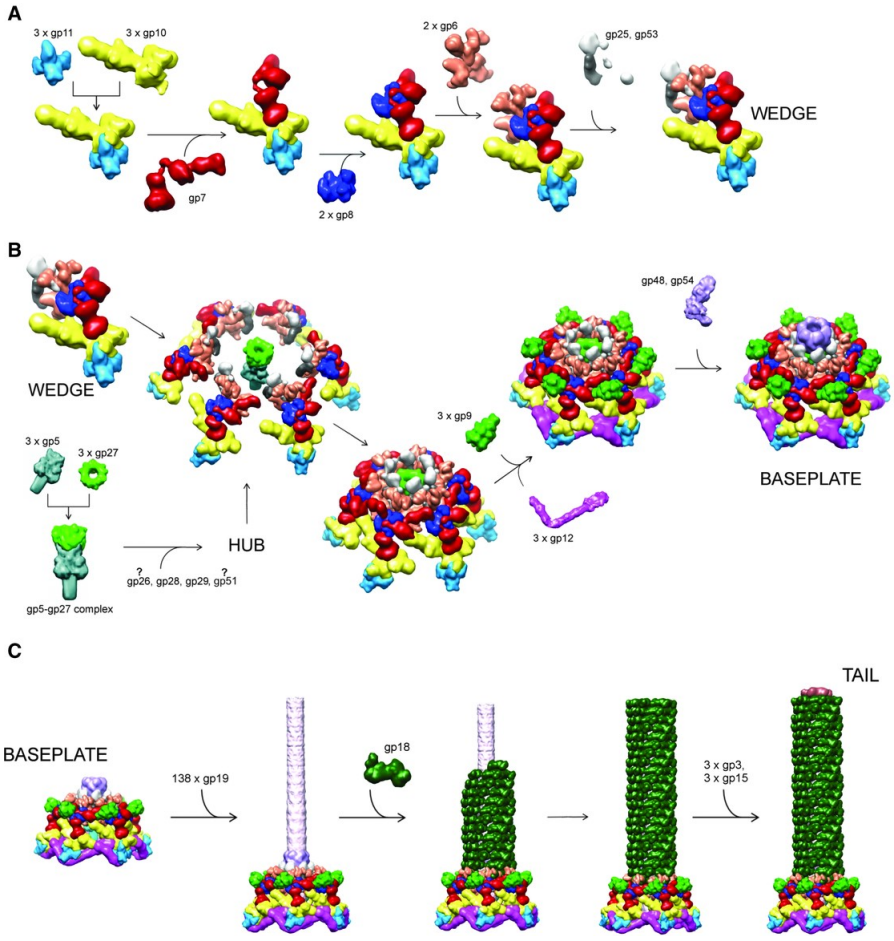

FIGURE 21.7 **Assembly of the T4 tail**. Assembly of the wedge **(A)**, the baseplate **(B)**, and the tail tube **(C)** with the sheath. (Reprinted from Leiman PG, Arisaka F, van Raaij MJ, et al. Morphogenesis of the T4 tail and tail fibers. *Virol J*. 2010;7:355. http://creativecommons.org/licenses/by/2.0.)

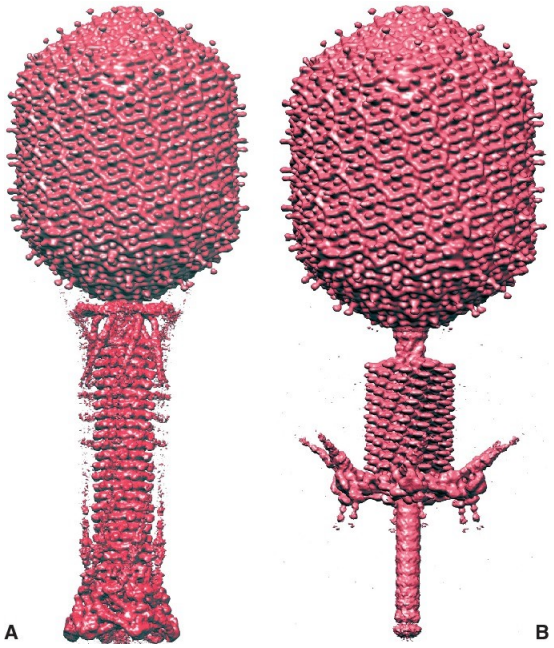

FIGURE 21.8 Cryoelectron microscopy (cryo-EM)-derived model of the T4 phage particle prior to **(A)** and upon **(B)** host cell attachment. Tail fibers are disordered in the cryo-EM structures, as they represent the average of many particles each having the fibers in slightly different conformations.

Assembly begins with the baseplate wedge, which is composed of seven different polypeptides (Fig. 21.8). The hub is independently assembled from gp5, gp27, and gp29 (the tail tape measure protein), which then joins with gp12, gp9, gp48, gp54, and six preassembled wedge components to form the baseplate, along with its short tail fibers (gp12). The tail tube (composed of 138 copies of gp19) assembles onto the baseplate, gp18 forms the tail sheath around the tube, and gp3 and gp15 cap the top of the tail. The long tail fibers are bent rods composed of trimers of gp34, gp36, and gp37 and a hinge composed of gp35. They are made by a separate pathway from the rest of the tail with the aid of gp38 and gp57A chaperones. Tail fibers normally attach to the baseplate only after heads and tails are joined and attach to the gp9 socket protein on the baseplate. Both the Wac whiskers and gp63, a protein that is also an RNA ligase, have been found to accelerate tail fiber addition. As a general rule, the assembly of the individual components of tails and tail fibers follows a strict order (with a few exceptions), such that omission of one component or protein halts assembly at the stage when that component is required.

Adsorption of T4 to *E coli* involves recognition of the outer membrane–located OmpC receptor by the long tail fibers, specifically the C-terminal tip of gp37. This association results in a large conformational change in which the hexagon-shaped baseplate opens up into a star shape, the sheath contracts, and the internal tube is pushed through the baseplate and through the cell envelope, with the lysozyme action of gp5 facilitating passage through the peptidoglycan layer. The tape measure protein gp29 is presumed to exit the tube so that DNA can be released from the capsid, through the tube, and into the cytoplasm of the cell. In this process, the baseplate and sheath essentially adopt two alternative configurations: a hexagonal baseplate with an extended sheath, and a “star” baseplate configuration with a contracted sheath. The transition from one to the other involves numerous dynamic changes, with perhaps the most prominent being the rotation of more than 100° of the gp10-gp11 complex that attaches the side tail fibers to the baseplate. As a consequence, the short tail fibers unfold from under the baseplate and extend toward the cell surface. A movie of a reconstruction of the whole T4 injection process is available at http://www.youtube.com/watch?v=41aqxcxsX2w.

Antigen Display

Because Hoc and Soc are dispensable for the formation of mature virions, they provide an intriguing platform for display of antigens and other proteins on the outside of the head shell. Fusion proteins containing the gp23 association sequences of Hoc or Soc attached to an antigen of choice can be expressed during infection of a Hoc- and Soc-defective mutant of T4 to generate decorated particles.[201] Alternatively, purified fusion proteins can be assembled onto *hoc*$^{-}$ *soc*$^{-}$ mature virions *in vitro*. The ability to add a large number of epitopes—up to 1895—onto a single virion is a particularly attractive feature.[126] These approaches have been successfully demonstrated for antigens of a variety of pathogens including human immunodeficiency virus (HIV), poliovirus, swine fever virus, foot-and-mouth virus, and *Neisseria meningitides*.[172,186] Some of these show considerable promise as vaccine candidates, including for protection against anthrax and SARS-CoV-2.[157,229]

This approach for virion decoration has also been applied to the construction of small peptide libraries and using these to identify associating proteins. In one example, libraries of the T4 large terminase subunit were panned and a peptide identified that interacts with the late sigma factor, gp55, and subsequent analysis showed that gp55 plays a role in loading the terminase onto the viral genome.[131]

Other Tailed Phages

The *E coli* phages T4 and λ are the most extensively studied individual phages of the ~10^{31} on the planet, and they have shown the way over the past approximately seven decades to a remarkable number of fundamental insights into how viruses earn their livings and interact with their hosts, not to mention revealing some of the most basic aspects of molecular biology. However, another lesson from studies of tailed phages is that they are astonishingly diverse, both in their genetic content and in the genetic and biochemical mechanisms they have developed. Here we present a small selection of other well-studied tailed phages, emphasizing only features of their life cycles that are not found in λ or T4.

Bacteriophage HK97

Escherichia coli phage HK97 was a relatively unknown distant relative of phage λ, but it was selected for further study because it morphologically resembled λ but has a longer tail.[93] However, HK97 has become an important model system for capsid assembly and structure because of a relatively simple capsid assembly pathway that is experimentally amenable (Fig. 21.9A). HK97 requires only three proteins to direct capsid assembly of its T = 7 capsid: the portal protein, the protease, and the major capsid protein. Missing from this list is the scaffolding protein identified as a required assembly factor in all tailed phages studied previously. In HK97, the scaffolding protein role is played by an ~100 residue N-terminal extension of the major capsid protein called the delta domain. Like the scaffolding proteins of other phages, the delta domain is removed after assembly by the HK97 capsid maturation protease, and the digested pieces exit from the particle. This proteolysis makes the assembly essentially irreversible and results in a particle (prohead II) that is ready for DNA packaging; subsequent conformational changes lead to the expansion of the particle from the round prohead shape to the larger angular shape of the mature form, head II.[52]

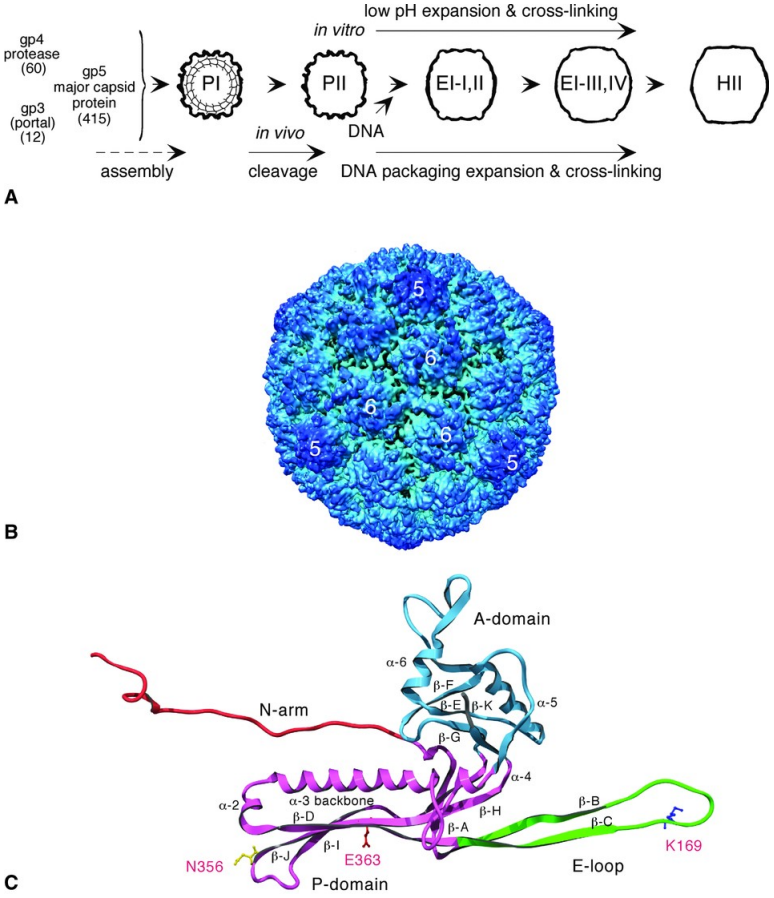

FIGURE 21.9 **Assembly of HK97**. **A**. PI is prohead I, the first intermediate, and PII is prohead II, which is the cleaved form after proteolysis. EI-I, EI-II, EI-III, and EI-IV are expansion intermediates, as detected in.[119] HII is the mature HK97 capsid containing fully cross-linked major capsid protein. Numbers in parentheses on the left are copy numbers of proteins used. **B**. Cryoelectron microscopy reconstruction of HK97 prohead I with three hexons labeled with a *6* and three pentons labeled with a *5*. (J. Conway, A. Steven, N. Cheng, R. Duda, and R. Hendrix, unpublished data.) **C**. Subunit F extracted from the head II structure (PDB ID 1OHG) showing major elements of the structure and the three residues involved in cross-link formation.

A particularly surprising aspect of HK97 virion assembly is how the mature capsid protein is stabilized, using covalent cross-links between the major capsid subunits.[217] This is in contrast to phages that use *decoration proteins*, such as λ and T4 (as described earlier), or phages such as P22 and T7, which achieve their stable mature forms by conformational changes alone. The covalent bonds in HK97 act like rivets between the inside and outside of the capsid, forming between a lysine—part of a secondary structure element on the outside of the capsid, the "E-loop"—and an asparagine on an inner part of a separate subunit. The reaction occurs during the conformational changes of maturation, which brings the two cross-link residues into a reaction center containing a third residue, glutamic acid, which is required to "catalyze" the reaction (Fig. 21.9C). There are 415 of these cross-links, which also change the overall topology of the capsid polypeptide chains into covalent circles that are interlocked in a way that resembles chain mail.[217] This results in an unusually stable capsid.

All of the capsid assembly intermediates illustrated in Figure 21.9 have had their structures determined by cryo-EM, so there is a wealth of information about the conformation changes that occur, at increasingly higher resolutions as techniques improve.[51,119] The very stable cross-linked HK97 mature capsid was the first of the tailed phage capsids to yield a structure by x-ray crystallography[217] (other HK97 intermediates have also yielded x-ray structures since). The fold of the subunits found in the HK97 capsid (Fig. 21.9C), dubbed the HK97 fold because it was unique among known tertiary structures at the time, has since been found in virtually all other tailed phage capsid proteins that have been studied so far, and also in the inner part of herpesvirus nucleocapsids. The x-ray models of HK97 combined with lower-resolution cryo-EM density maps have enabled a fairly complete description of the later stages of HK97 maturation,[93,119] and it is hoped that similar work on the earliest stages (Fig. 21.9B) will help complete the story and shed light on the mechanism of capsid size determination, which is still poorly understood.[66]

Bacteriophage T7

E coli phage T7 is one of the seven classical "type" virulent phages anointed by Max Delbrück as appropriate for study in the early days of phage molecular biology.[145] T7 is a strictly lytic phage with a short (~20-minute) life cycle; if T4 is the *Tyrannosaurus rex* of phages, T7 is the *Velociraptor*. The genomic DNA in the T7 virion is not permuted, and all the genes are transcribed in the same direction. Upon infection, only the first 7 kbp of the "left" end of the DNA, corresponding to the upstream end of transcription, enter the cell initially. This segment of DNA contains three strong, tandem promoters recognized by the host RNA polymerase, leading to transcription and expression of a small number of genes. These include a protein that inactivates restriction enzymes but, most importantly, includes the phage RNA polymerase. This is the highly efficient single-subunit RNA polymerase that has been used to drive expression in many plasmid protein expression vectors. For T7, its job is to transcribe the remainder of the phage genes, starting from a phage-specific promoter near the end of the short sequence of DNA that was initially injected into the cell. Remarkably, entry of the remaining 83% of the genomic DNA into the cell is driven by transcription by the phage RNA polymerase; as the polymerase transcribes in the direction of the DNA still in the phage capsid, its translocation along the template pulls the remainder of the DNA into the cell.[146]

In light of T7's long-standing reputation as a quintessentially lytic phage, it was a surprise when a T7-like prophage (!) was found in the genome of a *Pseudomonas putida* strain.[36] The prophage has an essentially complete inventory and arrangement of T7 family genes, with an extra ~4 kbp of DNA at the right end of the genome encoding an integrase and at least three additional proteins of unknown function. Unlike in the better-studied prophages, the gene order of the T7-like prophage is not circularly permuted relative to the gene order in the virion DNA. Like many other prophages, the T7-like prophage is integrated into the middle of a tRNA gene and appears to have restored an intact tRNA gene with a copy of the downstream half of the tRNA gene encoded at one end of the prophage.

Bacteriophage P22

The genetic diversity of the lambdoid phages is legendary among virologists, and nowhere is this more clearly demonstrated than in their head assembly genes. There are currently 12 types of lambdoid phages known whose head genes have diverged to the point where their major capsid protein amino acid sequences are not recognizably similar, and there are significant parallel variations in their head assembly mechanisms.[39] One of these types is exemplified by *Salmonella enterica* phage P22, a temperate phage with a 41 724-bp genome.[159] Its genes are organized and regulated like those of λ, but its virion has a very short tail (it is a member of the *Podoviridae* family; Table 21.1), and unlike λ it utilizes a headful packaging strategy. P22 was discovered at about the same time as λ, but it was immediately noticed that, unlike λ, P22 could transfer host genetic material from one *Salmonella* cell to another.[230] This process was called *generalized transduction* because any host gene could be transferred in this way. It is now known that the P22 DNA packaging apparatus occasionally makes a mistake and initiates packaging on the host chromosome instead of on its own phage DNA.[136] P22 is a headful packaging phage, and the headful nuclease that cuts the packaged DNA from the long packaging substrate DNA has little sequence specificity. So, when a head is full of host DNA, the headful nuclease cuts the packaged DNA from the host chromosome, normal tails assemble on these heads, and a "virion" is produced, which is in all respects identical to a genuine P22 virion except that it contains a single ~43-kbp molecule of host DNA instead of the phage chromosome. Such a "transducing virion" can, like an authentic P22 virion, inject its host DNA payload into a susceptible *Salmonella* cell, but this DNA cannot replicate and does not encode P22 proteins. This would be a dead end of no consequence, except that the injected host DNA can replace the parallel portion of the new host's chromosome by homologous recombination that is mediated by host cell proteins.[136] Because P22 does not inject any toxic proteins with its DNA, this recipient cell can survive the experience, and if there are genetic differences between the original donor host and the new recipient host, these differences can be transferred from the donor cell to the recipient cell by this transduction process. Geneticists studying *Salmonella* soon found this to be an extremely useful way of moving specific genetic alleles from one *Salmonella* strain to another, and small terminase subunit mutants of P22 were found that had error-prone DNA packaging machinery that made the process much more efficient.[37] Soon generalized transducing phages had been isolated to perform this function for many bacterial species. For wild-type generalized transducing phages, virions with host DNA typically make up 0.1%-2.0% of the total virions made during an infection, but the frequency of transduction of any given allele from one host to another is considerably lower than this, because only a small fraction (1%-3%) of the transducing virions carry host DNA that contains any particular host DNA sequence. In addition, the efficiency of recombination of the injected host DNA with its resident homolog is much <100%.[136] Nonetheless, successful transduction frequencies as low as one in a million recipient cells can be utilized in the laboratory, and transduction remains a common and powerful tool of bacterial genetics. Generalized transduction certainly also occurs in nature, where it is thought to be responsible for much of the genetic exchange that happens between bacteria in the wild.

P22 was also the first phage found to encode an *antirepressor* or protein that antagonizes its prophage repressor.[199] P22 antirepressor protein binds the P22 prophage repressor and stops it from binding to its operator. In this way the presence of antirepressor can initiate prophage induction or prevent establishment of lysogeny. Expression of the antirepressor gene is controlled by two other P22 transcriptional repressor genes (*mnt* and *arc*) and a small RNA gene (*sar*).[127] The Mnt repressor binds its operator, which overlaps the antirepressor gene's promoter, and keeps transcription from initiating there in the prophage state. However, if the Mnt repressor releases its operator, antirepressor protein is made, and it in turn inactivates the prophage repressor so that prophage induction ensues. The small *sar* RNA, which blocks translation of antirepressor mRNA, and both Mnt and Arc transcriptional repressors are all required to lower antirepressor synthesis to the very low level required for P22 to establish and maintain the lysogenic state. The *sar* RNA was one of the first untranslated regulatory RNAs to be discovered and characterized.[127] Antirepressors of several types are now known to be fairly commonly encoded by temperate phage genomes.

Some tailed phage virions utilize polysaccharide receptors to adsorb to the bacterial surface. P22 is such a phage, and it was instrumental in the discovery that such virion receptor-binding proteins (tailspikes) are usually also enzymes that cleave these surface polymers. P22 virions have 6 trimeric tailspikes and so have 18 polysaccharide binding/cleavage sites. The detailed role of this cleavage is not completely understood, but it appears to allow the virion to work its way down to the outer membrane surface.[41] Such tailspikes are very specific for the particular sugars that make up the polysaccharide chain, which means that these phages are very specific for particular bacterial subspecies, strains, or "serovars." P22, for example, only adsorbs to a small fraction of *Salmonella* cells, those whose O-antigen polysaccharides are built with a mannose-rhamnose-galactose repeat. This specificity of P22 tailspike binding can be used to identify its host bacterium, *S enterica* serovar Typhimurium.[202] Phages that infect *Enterobacteriales* bacteria (the order that includes *Escherichia*, *Shigella*, *Salmonella*, *Serratia*, etc.) are known that carry similar tailspikes with at least 51 different polysaccharide specificities.[42] All these phages are most likely similarly restricted to infecting a small subset of their host species, and the surface polysaccharide of their hosts could presumably be identified in a similar manner. Some phage virions carry more than one tailspike, and they can adsorb to and infect multiple hosts that have surface polysaccharides to which

their different tailspikes bind.[189]

The genetic diversity and mosaicism of the phage P22 virion assembly genes have been particularly well studied. These phages have been evolving for a very long time, and even in a phage group as narrowly defined as the P22-like lambdoid subgroup, the coat proteins have diverged *within* this group to the point that some pairs are <15% identical in amino acid sequence, apparently without genetic contact with coat genes from other tailed phage types.[156] Other proteins involved in recognition of DNA to be packaged and in receptor binding are even more variable (see earlier), and in these two cases there has been horizontal acquisition of some gene types from other more distantly related tailed phage groups.[42] In addition, there has been extensive genetic exchange among the various phages *within* the P22-like group, and in many cases parts of genes that encode protein domains rather than whole genes have been exchanged.[42]

Recently phages are being exploited for nanotechnology purposes, and P22 has been in the forefront of this research. The facile P22 genetic system has allowed P22 virion and procapsids to be specifically coated with and/or filled with foreign materials to create nanoparticles with various potential uses.[190,191] Particularly attractive is the use of controlled *in vitro* capsid assembly using cargo-fused scaffold protein for efficient and well-controlled encapsulation of both and small molecules.[191] This represents a potentially new nanoscale platform with multiple applications in biomedicine.

Bacteriophages P2 and P4

Bacteriophage P2 is the best-characterized member of a large family of temperate *Myoviridae* found in the genomes of γ-*Proteobacteria*.[152] It was isolated in 1951 by Bertani[20] from the oldest known lysogen, the Lisbonne and Carrère strain of *E coli*, along with phages P1 and P3. One hallmark of P2-related phages is their lack of ultraviolet (UV) inducibility; unlike the lambdoid phages, repressors of P2-related phages are not cleaved in the presence of activated RecA.

P2 is among the smallest *Myoviridae*, with a genome of 33 593 bp; the virion DNA is linear and double stranded, with 19-bp cohesive ends. DNA replication proceeds by a modified rolling circle mechanism reminiscent of that used by single-stranded DNA phages such as φX174, and like φX174, P2 depends on the host *rep* helicase.[152] Replication requires a *cis*-acting phage-encoded initiator protein, the A protein, that catalyzes cleavage at the origin and becomes covalently linked to the 5′ end of the cleaved strand. It acts again at the end of replication to cleave the newly synthesized origin and rejoin the displaced old strand. The resulting products are monomeric double-stranded circles, an unusual substrate for packaging of double-stranded phage DNA but one that is required for the P2 packaging reaction.[22]

All P2 transcription is carried out by the host RNA polymerase. As in other temperate phages, early transcription initiates from a pair of divergent promoters encoding the lysogeny and replication functions. The P2 immunity repressor, C, differs from the majority of phage repressors in that it recognizes nonpalindromic direct repeats of DNA, even though it is structurally similar to repressors that bind to inverted repeats. P2 C regulates its own promoter and blocks expression of the rightward operon encoding the replication genes and *cox*, which encodes the repressor of the lysogenic promoter. P2 Cox is unusual in that it functions not only as the repressor of the lysogenic promoter but also as the recombination directionality factor for prophage excision.[226]

Transcription of the P2 late genes requires the product of the phage *ogr* gene, a transcriptional activator that binds to a conserved sequence about 55 bp upstream of the initiation sites for the four P2 late promoters.[47] Ogr was the earliest example of a transcription factor that interacts with the α subunit(s) of *E coli* RNA polymerase. It is a small zinc-binding protein found almost exclusively among P2-related phages and their satellites, with a Cys_2Cys_2 motif essential for metal binding and activity.

Capsid assembly has been studied extensively in P2, largely in the context of its relationship as a helper phage for satellite phage P4, which is discussed in more detail later. P2 has also served as a model for bacteriophages with contractile tails. An exciting recent development was the demonstration that the bacterial membrane–penetrating protein, gpV, is an iron-binding spike.[24]

P4 was discovered in the early 1960s by Erich Six and was initially characterized as a satellite bacteriophage dependent upon a P2 helper phage for lytic growth. Subsequent work has shown that P4 may be more appropriately considered to be an integrative plasmid that acquired the ability to exploit a helper phage for highly specialized horizontal transfer.[152] In the absence of a helper genome, P4 can be maintained as an integrated prophage or as a multicopy plasmid. P4 replicates its DNA by θ mode both in the multicopy plasmid state and during the lytic cycle, and this replication is independent of helper phage functions. P4 immunity is regulated by a novel mechanism involving transcription termination caused by sequence-specific binding of a short stable immunity RNA. This immunity RNA is processed out of the same transcript that it regulates.

Exploitation of P2 by P4 can occur in several situations, including P4 infection of a P2 lysogen, P2 infection of a strain carrying P4 in either the immune-integrated or multicopy plasmid state, and co-infection by both phages. In each of these cases, P4 responds to the presence of the helper phage by activating functions that allow it to modify P2 gene expression appropriately. The nature and timing of the regulatory cross-talk depends on the infection conditions and appears to be designed to optimize P4 reproduction. One set of reciprocal regulatory interactions between P4 and P2 allows mutual derepression. The P4 Epsilon protein binds to the P2 immunity repressor and interferes directly with its function. Derepression of P4 by P2 requires a third activity of the P2 Cox protein, which stimulates transcription of the operon encoding the P4 replication functions from a promoter that bypasses the P4 immunity system. A second set of reciprocal interactions regulates late gene transcription. The two P4 late promoters are activated by P2 Ogr, the same protein required for P2 late transcription. One of the P4 late gene products, Delta, is an Ogr homolog that activates the same two P4 late promoters and the four P2 late promoters.[103] This mutual transactivation allows earlier expression of P4 late genes in the presence of a P2 helper. It also allows P4 to activate directly the transcription of the P2 morphogenetic genes required for packaging and lysis, bypassing their normal requirement for P2 replication.

The P4 lytic cycle requires all of the morphogenetic functions of the helper phage. A striking feature of the P2-P4 interaction is the redirection of the viral capsid assembly pathway. The 33-kb P2 genome is packaged into icosahedral, isometric T = 7 capsids with a 60-nm diameter. Satellite phage P4 packages its smaller (11 624 bp) genome into a T = 4 particle formed by the interaction of the P4-encoded size determination protein, Sid, with the P2 major capsid protein, gpN. The P2 scaffolding protein, gpO, is an internal scaffold required for assembly of the larger T = 7 P2 procapsids. Both P2 and P4 require gpO for capsid maturation; in addition to its role as a scaffold, it also acts as a protease for N-terminal processing of both gpN and itself. P4 Sid functions as an external scaffold, forming a size-restricting cage that influences the bending of the phage capsid protein at a flexible hinge region. Mature P4 virions have lost the external Sid scaffold but contain a P4-encoded decoration

protein, Psu, that helps to stabilize the smaller capsid. The smaller P4-induced capsid cannot accommodate the P2 genome, leading to interference with helper phage growth.[152]

Recently this phenomenon has been found to be much more prevalent than was previously thought, and numerous "satellite-helper" phage relationships have been discovered in which bacterial regions call pathogenicity islands (chromosomal regions that contain bacterial virulence genes) trick phages into packaging their genes into virions. This "molecular piracy" is an important mechanism for the spread of virulence genes.[65,74] Notable examples are the *Staphylococcus aureus* Pathogenicity Islands (SaPIs), a large family of chromosomal islands expressing toxins including the toxic shock syndrome toxin.[128]

Bacteriophage G

Bacillus megaterium phage G is perhaps the largest lytically growing phage known and among the nonphage viruses is exceeded in size only by the Mimiviruses. The G genome is 497 513 bp long, with nearly 700 predicted protein genes and 17 tRNA genes.[90] Fewer than one-third of the predicted protein sequences match any sequences in the databases. The sequences with good database matches include some virion structural proteins, DNA replication functions, and a wide variety of metabolic enzymes. Compared to phages with smaller genomes, phage G has an unusually large number of apparent transcription factors and even has some translation components, including the tRNAs and an apparent tRNA synthetase, features that have also been reported for the very large eukaryotic Mimiviruses. Metagenomic analyses have identified even larger phagelike sequences ("huge" phages) although these have yet to be propagated lytically.[6]

Bacteriophage Mu

Escherichia coli phage Mu (*Mu* is short for "Mutator" and is not the Greek letter) is a temperate phage that gets its name from the fact that the prophage integrates at essentially random positions around the host chromosome, often inactivating genes into which it inserts.[154] Mu is a transposon as well as a bacteriophage, and when it grows lytically it replicates its DNA by replicative transposition, resulting in ~200 copies of the phage genome inserted around the host chromosome. These phage genomes are excised from the host DNA and packaged into procapsids in such a way that some flanking host DNA is included in the DNA in the virion— 50-150 bp on the left end and 1-2 kbp on the right end. In this sense the Mu genome is always inserted between flanking host sequences, whether it be in the virion particle, as a prophage, or replicating lytically.

Another striking and unusual feature of Mu, shared with only a minority of the extensive family of Mu-like phages as well as with some members of the P1-like and P2-like phage families, that has been described is that it has two alternative types of tail fibers, which give it two alternative host ranges. It achieves this by having a 3-kb invertible segment of DNA (the "G segment") containing sequences that encode two alternative C-terminal halves of the tail fiber, one on each end of the G segment.[26] The orientation of the G segment in the genome determines which of these alternative C-terminal half-genes is fused to the half-gene located immediately adjacent to the upstream side of the G segment encoding the N-terminal half of the tail fiber. The N-terminal half of the tail fiber attaches the fiber to the phage tail and the C-terminal half contains the host cell receptor binding specificity, determining the host range. Inversion of the G segment is mediated by a DNA invertase, Gin, encoded within the G segment. Gin is a member of a family of invertases, including the Hin invertase of *Salmonella*, which is responsible for flagellar antigen switching.

Bacteriophages P1 and N15

Just as phage Mu is both a bacteriophage and a transposon, *E coli* phages P1 and N15 are both bacteriophages and plasmids. N15 is a temperate phage with a 46 363-bp genome that has many features in common with phage λ. P1 is a different type of temperate phage whose genome is considerably larger (93 601 bp). In the lysogenic state these two phages do not integrate their DNA into the host chromosome. The P1 and N15 prophages replicate as linear and circular plasmids, respectively.[171,224] P1 is a generalized transducing phage and a classical tool of *E coli* genetics,[136] and it also played a critical early role in the discovery of restriction endonucleases (the P1 genome encodes such an enzyme), which in turn was critical in the early development of genetic engineering.[49]

The P1 and N15 prophage plasmids are both present in low copy number close to the number of bacterial chromosomes in the cell, and the study of P1 was important in our current understanding of how bacterial plasmids are partitioned into the two daughter cells during cell division.[68] Both phages encode two proteins, ParA and ParB, that are responsible for accurate plasmid segregation. ParB molecules bind to a centromerelike site(s) on the phage DNA to form a large macromolecular structure, and ParA protein is an ATPase that can form filaments and binds to ParB. The precise mechanism of control of plasmid partitioning by these proteins is not yet known, but it has been speculated that polymerization of ParA into filaments acts to push or pull the plasmid DNAs into the daughter cells.[68] In spite of the fact that P1 does not integrate its prophage into the host chromosome, it encodes a site-specific DNA recombinase, Cre protein, that performs several roles for P1, including aiding prophage plasmid segregation by resolving dimer circles (that form by homologous recombination between two monomers) into two monomer circles.[224] The Cre/*lox* recombination system has been applied to the genetic engineering of higher organisms with great success, where it is used to cause gene targeting or deletion to occur in response to a specific environmental signal or to occur only in a specific tissue.[187]

Most bacterial DNAs are circular, so the fact that the N15 prophage plasmid is a linear dsDNA molecule is unusual. At the ends of this plasmid the two strands are joined covalently to form a closed hairpin at each end. The mechanism by which the N15 plasmid prophage is formed is interestingly analogous to the mechanism of λ prophage integration. The DNA in the N15 virion, like λ virion DNA, is linear dsDNA with 12 base ssDNA cohesive ends that join upon DNA injection to circularize the molecule. When the phage enters the lysogenic cycle, this circular form of the DNA is converted into the linear prophage form by integrase in the case of λ and by a phage-encoded enzyme known as protelomerase ("prokaryotic telomerase") in the case of N15.[170] Protelomerase binds to a *tel* site adjacent to its gene, analogous to λ's *attP*, and cuts both strands of the DNA. But instead of joining the cut ends to host DNA, it joins the 5′ end of each strand to the 3′ end of its complementary strand, thus forming the hairpin ends of the linear prophage plasmid.[99] Replication of the linear N15 plasmid then proceeds as normal semiconservative duplication by separation of the two parental strands and synthesis of the complementary strand without breaking the parental DNA chain. This converts the linear molecule into a dsDNA head-to-head dimer circle, which is in turn resolved into two hairpin-ended

linear molecules by action of the protelomerase at the two *tel* sites in the dimer circle.[170]

Bacteriophage ø29

The *Bacillus subtilis* bacteriophage ø29 is a small, dsDNA lytic virus of the *Podoviridae* family. Although λ and T4 were being investigated intensively in the mid-1960s, ø29 was chosen for study because it is small (~20 genes) yet shares some of the complexities of other larger phages, including a prolate (elongated) head and a complex, noncontractile tail.[7] From the start, the study of ø29 held the promise of understanding the function of every gene and of isolating and characterizing every intermediate of the assembly pathway. The system was deemed well suited to investigate complex questions such as the mechanism of form determination, the regulation of assembly *via* sequential protein and DNA conformational changes, and the mechanism of DNA packaging. ø29 has since proven to be a rich model system, especially for the study of DNA replication and the DNA packaging phase of viral assembly.

The genome of ø29 is a linear, unit-length (ie, nonconcatemeric) chromosome of ~19.3 kbp.[140,182] The ø29 chromosome is unusual in that a phage terminal protein, gp3, is covalently bound to each 5′ end of the DNA via a phosphoester bond. Terminal proteins are also found in the chromosomes of some other phages, such as PRD1 and the eukaryotic adenoviruses. These proteins are essential for DNA replication, which proceeds by a protein-primed mechanism. In ø29, a specific serine residue of gp3 provides the free 3′-OH needed to initiate DNA replication. Replication is bidirectional, with the viral-encoded polymerase, gp2, forming a complex with a free gp3 at both ends of the DNA and then catalyzing covalent attachment of a 3′-deoxy-adenosine monophosphate (dAMP) to the terminal protein. The complex “slides back” to the first nucleotide, and DNA synthesis proceeds from this single priming event. This mechanism allows ø29 to overcome the challenge of replicating the ends of linear DNA without loss of genetic information. The monomeric phage-encoded DNA polymerase is notable in that it has been shown to have exceptionally high processivity, and it is capable of both polymerization and template-strand displacement, precluding the need for a helicase to unwind the template DNA.

During dsDNA phage virion assembly, the genomic DNA is packaged into a preformed protein shell (prohead or procapsid).[168] This process is driven by an ATP-dependent molecular motor that transiently assembles at the unique portal vertex of the head. In most phages, the motor consists of the connector and a terminase complex composed of large and small subunits that house the ATPase/nuclease and DNA recognition functions, respectively. In ø29, the covalently bound terminal proteins are also essential for packaging,[147] likely providing the role of the small terminase subunit. The ø29 packaging ATPase is smaller (~60%) than other phage large terminase subunits, in part due to the lack of a required nuclease function as the packaging substrate is already unit length. Additionally, ø29 is unique in that a small noncoding RNA is also an essential component of the motor. This 174 nucleotide prohead RNA (termed pRNA) forms a novel pentameric ring via intermolecular base pairing.[147] The pRNA ring serves to bridge the connector and the ATPase rings in the packaging motor.[64,80] Because all the dsDNA phages are thought to employ similar packaging mechanisms, the functions of pRNA likely reside in subdomains of the larger motor proteins of other phages[168] (Fig. 21.10). Movement of DNA into the head is driven by the ATPase gp16. Like the analogous large terminase enzymes of other dsDNA phages, this ATPase ring is related to a large class of cellular ring ATPases that perform many substrate translocating functions.[28]

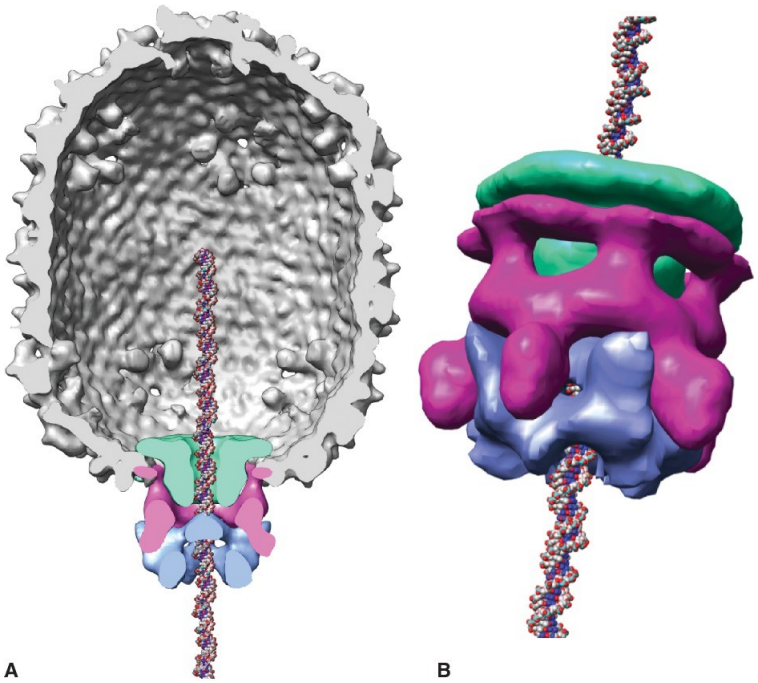

FIGURE 21.10 **Packaging of φ29 DNA**. The φ29 DNA packaging motor. **A**. Cutaway view of cryoelectron microscopy reconstruction of the prohead/motor complex showing the molecular envelopes of the motor components.[128] Gray is the capsid, green is the head-tail connector, magenta is the prohead RNA (pRNA) ring, and blue is the packaging ring adenosine triphosphatase (ATPase) gp16. A DNA model has been placed in the channel for reference. (Courtesy of Marc Morais. **A** reprinted from Grimes S, Ma S, Gao J, et al. Role of φ29 connector channel loops in late-stage DNA packaging. *J Mol Biol*. 2011;410(1):50-59. Copyright © 2011 Elsevier, with permission.) **B**. Close-up of the motor components. (**B** adapted with permission from Ding F, Lu C, Zhao W, et al. Structure and assembly of the essential RNA ring component of a viral DNA packaging motor. *Proc Natl Acad Sci U S A*. 2011;108(18):7357-7362.)

A ring motor can work using a mechanism where each subunit works independently, or alternatively, two or more motor subunits can work together via a more complex and coordinated mechanism. Single-molecule laser-tweezers studies indicate that DNA translocation subunits are

coordinated such that the ring loads with ATP while holding the DNA during a “dwell” phase, then hydrolyzes these ATPs to drive a “burst” of 10 base pairs into the head that is coupled to the release of inorganic phosphate from the motor. This high degree of coordination requires communication between motor components, possibly mediated through adjacent ATPase subunits, the pRNA ring to which it is bound, and/or the DNA substrate itself.

Studies of ø29 and other phage motors have revealed that the packaging motor is powerful when compared to other biological molecular motors, generating forces over 65 piconewtons, which is approximately an order of magnitude stronger that skeletal myosin.[168,192] In part this high-force-generating capacity is required to overcome the resistance encountered when compacting the highly charged and relatively stiff DNA to near-crystalline density inside the head.

Insights from other Phage Families

Tectiviridae: dsDNA, Linear, With Proteins on 5′ Ends

Tectiviruses, typified by *E coli* phage PRD1, share with the tailed phages (*Caudovirales*) a linear dsDNA genome in the virion as well as a few general features of their infection strategy, but in many ways they are quite distinct. Perhaps the most prominent difference is that the tailless PRD1 virion contains a lipid membrane, located inside an icosahedral protein capsid and surrounding the viral DNA[79] (Fig. 21.11). Its linear DNA is 14 927 bp long, not permuted, with inverted terminal repeats at the ends. Thirty-seven genes have been identified that are arranged in five operons. Promoters for the two early operons are in the terminal repeats, pointing toward the middle of the DNA, and the three late transcripts fill the center of the map, all oriented in the same direction.[79] The 5′ ends of the two DNA strands are covalently joined to a phage-coded protein that serves as the primer for DNA replication by a phage-coded polymerase.

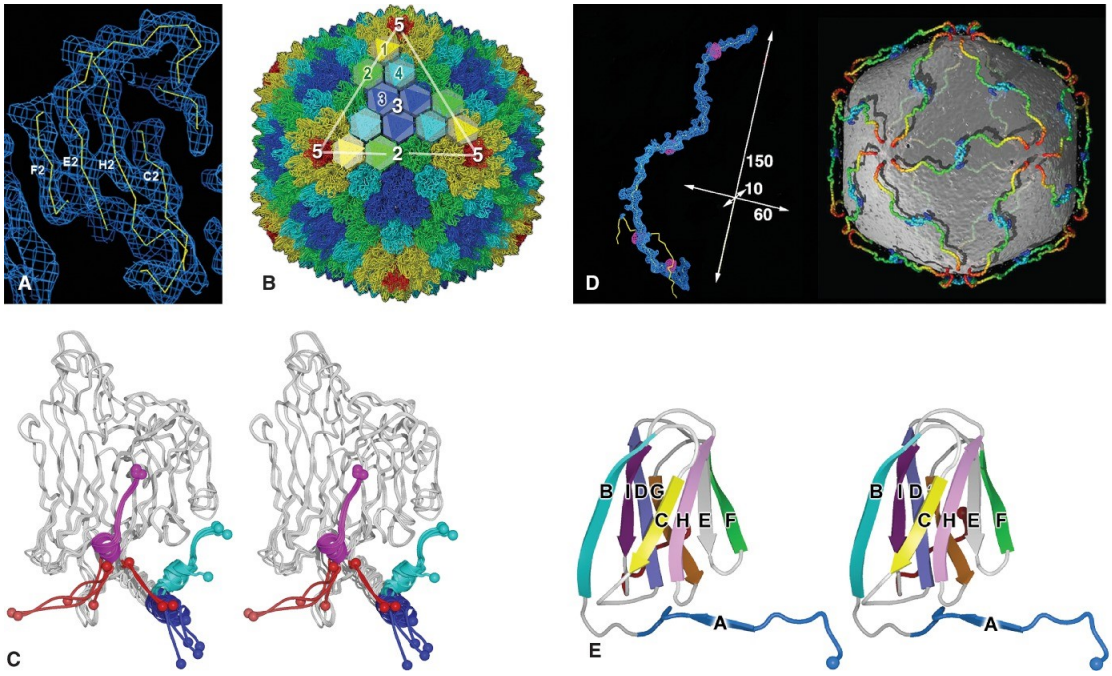

FIGURE 21.11 **Architecture and structural components of bacteriophage PRD1**. PRD1 is composed of four protein subunits P3, P16, P30, and P31, surrounding a membrane. P30 is a linearly extended protein that nucleates the formation of the icosahedral facets (composed of trimers of the major capsid protein, P3) and acts as a molecular tape measure, defining the size of the virus and cementing the facets together. Pentamers of P31 form the vertex base, interlocking with subunits of P3 and interacting with the membrane protein P16. **A**. Strands of the second of two jelly-roll domains in the PRD1 capsid subunit, P3. **B**. The building blocks of a facet (the triangular area defined by white lines). The icosahedral asymmetric unit contains 12 P3 subunits arranged as four trimers, with orientations represented by triangles labeled 1-4 and colored yellow, green, blue, and cyan, respectively (the underlying gray hexagons show the trimer morphology; numbers and symbols follow the adenovirus convention). P3 trimers outside the marked facet are colored similarly and shown as coil, as is protein P31, drawn in red. **C**. Stereo Cα traces of the superposed 12 unique copies of major capsid subunit P3, showing switching at the N (*blue* and *cyan*) and C (*red* and *magenta*) termini. **D**. Protein P30 is shown on the left in *royal blue*, with the SeMet differences in magenta. Part of a twofold related P30 subunit is shown in *yellow*. Dimensions are in angstroms. On the right, 60 copies of P30 (colored *blue* through *green* to *red* from the N-terminus to the C-terminus) wrap around the electron density of the membrane. The small holes in the membrane close to the fivefold axes are the transmembrane helices of protein P16. **E**. Stereo view of P31. Jelly-roll strands are labeled B-G; the β-strand at the N-terminus is labeled A. N-terminal and C-terminal segments are colored blue and red, respectively. (Adapted by permission from Nature: Abrescia NG, Cockburn JJ, Grimes JM, et al. Insights into assembly from structural analysis of bacteriophage PRD1. *Nature*. 2004;432(7013):68-74. Copyright © 2004 Springer Nature.)

The high resolution x-ray structure available of the entire virion reveals several noteworthy features of PRD1 structure.[2] The major capsid protein, also known as P3 or hexon protein, has two β-barrel domains, each structurally similar to the single β-barrel domains that are found in many virus capsids. P3 trimerizes into a “hexon” that has a nearly sixfold symmetric arrangement of β-barrel domains and occupies the position in the capsid lattice that would be occupied by a hexamer in a canonical Caspar-Klug icosahedral structure. A different protein occupies the icosahedral vertices as a pentamer. These hexons and pentons are arranged in a pseudo T = 25 structure. There are two additional proteins forming the spike structure at the fivefold symmetry position but displaying symmetry mismatch. The spikes are involved in receptor recognition. One of the 12 vertices is a portal for the DNA packaging process where the DNA enters an empty procapsid through the protein and

membrane layers of the virion.[195] The virus release utilizes similar mechanisms to those operating in tailed bacterial viruses where a holin protein makes an opening to the plasma membrane and a lytic enzyme digests the peptidoglycan layer leading to cell disruption and virus release.

Microviridae: ssDNA, Circular

The *Microviridae* are small viruses with icosahedral capsids that contain the genome in the form of a circular ssDNA molecule. They are typified by the *E coli* phage φX174, genome size 5386 nucleotides, with which most of the experimental work with this group has been done.[71] A few tens of rather close relatives of φX174 have been isolated on enterobacterial hosts, and these have been used in comparative experimental studies with φX174 as well as in evolution studies.[216] A second small group of isolates infects hosts of the *Chlamydia*, *Bdellovibrio*, and *Spiroplasma* genera of bacterial hosts. This group of viruses shares general features with the φX174 group but differs in details of gene content and organization and of virion morphology; they are accordingly classified as a separate subfamily of the *Microviridae* family. Little has been known until recently about the abundance of the *Microviridae* in the environment. However, metagenomic studies of environmental samples have shown (when the sequencing technology employed can detect ssDNA) that *Microviridae* sequences are a significant component of the sequences in these samples that are recognizably of viral origin.[204]

Infection by φX174 begins with binding of the virion to the surface of the *E coli* host, and electron micrographs of infecting virions show them at locations where the inner membrane and the cell wall of the host are closely apposed. The initial receptor on the host is a portion of the lipopolysaccharide, recognized by a glucose-binding site on the virion surface. Host range mutants of the phage map to a different part of the virion surface, suggesting that there is at least one more host receptor molecule. It is not known how the virion DNA crosses the cell envelope to enter the cytoplasm, but it appears that the virion proteins remain outside the cell.

The virion ssDNA is of the plus sense, so transcription to produce mRNA cannot start until replication of the DNA has produced the minus-strand template. In fact, the first stage of DNA replication, which converts the single-stranded circular virion DNA into a double-stranded circle, is carried out entirely by host replication proteins. The resulting double-stranded circle, called RF I (replicative form I), can serve as the template for transcription but also serves as the template for the second stage of replication, in which the dsDNA RF I circles are amplified to make more dsDNA circles, now called RF II.[196] In addition to the host replication proteins required for converting virion DNA to RF I, this second stage of replication requires a phage-coded protein, the A protein, and an additional host protein, the *rep* helicase. The A protein binds to the replication origin, cuts the plus strand, and attaches the 5′ end of the plus strand to itself by an ester bond. The *rep* helicase then unwinds the 5′ end and replication proceeds by elongation of the free 3′ end of the plus strand. This is the classical rolling circle form of DNA replication, first identified in this phage. When the free single-stranded plus strand reaches unit length, the A protein cuts again and ligates the ends of the ssDNA to form an ssDNA circle. This ssDNA circle is identical to the virion DNA, and it is converted to the dsDNA circle, RF II, by the same host replication proteins that converted virion DNA to RF I.

The final stage of replication also uses a rolling circle mechanism initiated by the action of the phage-coded A protein, and the ssDNA plus-strand circles are produced as described for the second stage of replication. However, in this stage, replication takes place in a complex with the newly assembled procapsids. Formation of this complex is mediated by the phage-coded C protein. The ssDNA is apparently inserted into the procapsid as it is displaced from the rolling circle, with the packaging driven by the energy of replication (Fig. 21.12). Lysis involves a mechanism that alters the host MraY protein to interfere with cell wall synthesis.[17]

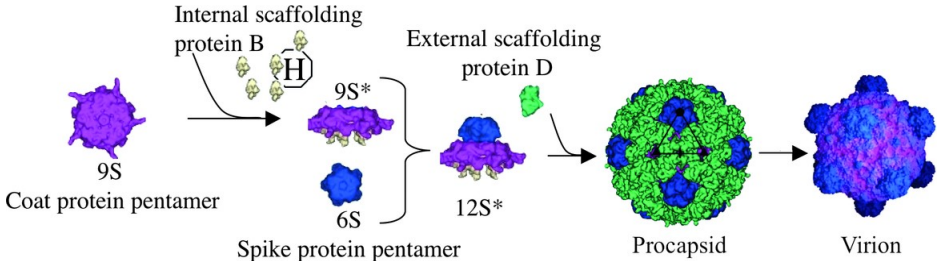

FIGURE 21.12 **Assembly of φX174 particles**. The first identifiable assembly intermediates are pentamers of the viral coat F and major spike G proteins, the 9S and 6S particles, respectively, which can form in the absence of both scaffolding proteins. Five internal scaffolding B proteins bind to the underside of the 9S particle, yielding the 9S* intermediate. This interaction induces a conformational change that allows 9S*-6S particle associations, forming the 12S* intermediate. The internal scaffolding protein also facilitates the incorporation of the DNA pilot protein H. Two hundred and forty copies of the external scaffolding D proteins, most likely in the form of 60 tetramers, associate 12 12S* particles into the procapsid, a DNA-less particle. Both scaffolding proteins are removed from the procapsid when particles are filled with single-stranded DNA (ssDNA) during the last stages of the maturation.

Inoviridae: ssDNA, Filamentous

Filamentous phages have striking morphologies, flexible long rods up to several micrometers in length, and diameters of only ~7 nm. These phages were first detected by Loeb[129] with phage f1, and later, M13 and fd were isolated, all of which are closely related and use *E coli* as their host. Several other *Inoviridae* have been described with host bacteria other than *E coli*,[212] but we will focus here on M13, perhaps the best studied of these phages.

M13 particles consist of a 6407-base single-stranded DNA molecule that is helically coated by about 2800 copies of a small (50 amino acids) major coat protein, gp8. The C-terminal sequence of gp8 is positively charged and interacts with the phosphate backbone of the DNA, stabilizing the phage particle. The tip of the phage has five copies each of gp7 and gp9, two minor coat proteins, whereas the base of the phage is made up of five copies each of gp3 and gp6 that are involved in adsorption to the host.

M13 adsorption to its host is quite distinct, is male specific, and uses *E coli* F pili for attachment. The N2 domain of gp3 binds to the tip of the F pilus and induces a retraction signal in the pilus similar to that induced by a contact with a female cell in bacterial conjugation. The F pilus

retracts by sequential disassembly into the bacterial inner membrane[29] along with the phage that effectively extends the tip of the pilus. During this process, the N1 domain of gp3 interacts with the periplasmic domain of the host TolA protein.[130] As the coat proteins partition into the membrane bilayer upon pilus retraction, the single-stranded DNA is released into the cytoplasm where it is converted into double-stranded DNA by the host DNA polymerase.

Only 11 gene products are encoded by the M13 genome, most of which are involved in assembly of the virion. The major coat protein gp8 is synthesized as a "procoat" with a cleavable signal sequence and inserted into the inner membrane of *E coli*. It is not a substrate of the signal recognition particle (SRP) and the Sec-translocase, but instead it requires the membrane insertase YidC for membrane localization.[183] After insertion, the procoat protein is converted to mature coat protein by the leader peptidase of *E coli*.[115] YidC is also involved in the membrane insertion of gp9.[163] All the virion minor proteins, gp3, gp6, gp7, and gp9, are membrane proteins, as are gp1 and gp11.[83]

Replication of M13 DNA proceeds by the rolling circle mechanism.[97] The endonuclease gp2 creates the free 5′ ends that are required to form new single-stranded circles. These are first converted to dsDNA to increase the numbers of rolling circles, and later in infection, when the structural phage proteins are synthesized, the newly replicated ssDNA is covered by the phage-encoded ssDNA binding protein, gp5.

As early as 9 minutes postinfection, the first progeny are released from the host cells without lysis (Fig. 21.13). Virion assembly takes place at the host membrane, initiating with gp7 and gp9 to form the tip of the prospective virion. This likely occurs within a transmembrane complex containing the membrane proteins gp1, gp11, and thioredoxin.[169] The ssDNA is then introduced into the assembly process by binding to the "packaging signal" in the M13 genome.[181] Oligomers of the major coat are then added to form a fivefold helical structure around the ssDNA that grows out from the cell surface.[151] During this elongation process ssDNA moves through the membrane and is simultaneously covered by major coat protein subunits, replacing the bound gp5 molecules. Passage through the outer membrane is facilitated by a porinlike structure containing gp4.[180] Finally, the assembly process is terminated by attachment of gp3 and gp6 to the end of the phage. Thus, the length of the virion filament is determined by the length of the ssDNA genome.

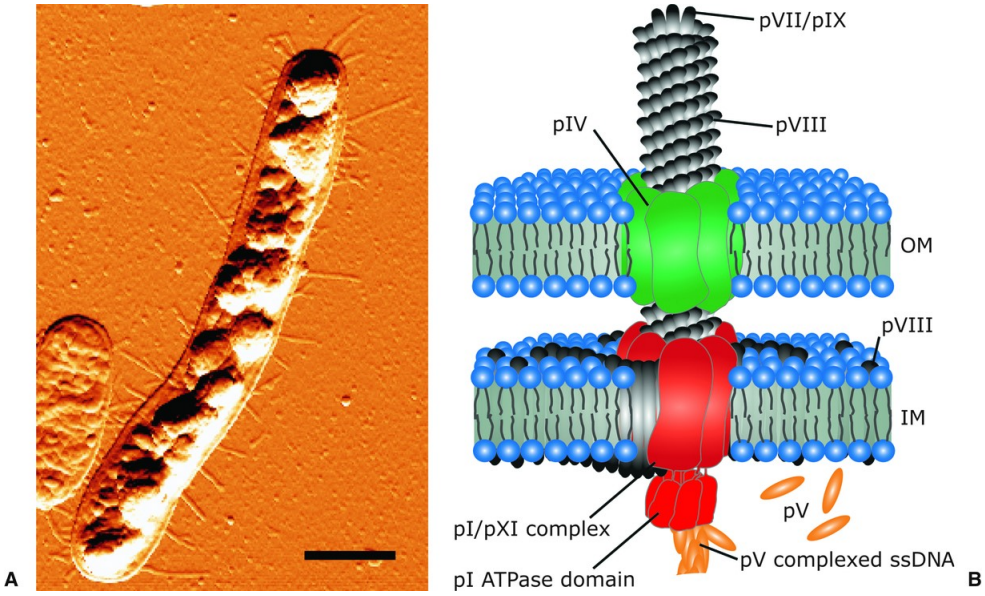

FIGURE 21.13 **Secretion of M13**. **A**. M13 phage secreted from *Escherichia coli* cells 9 minutes postinfection by atomic force microscopy. The bar represents 1 μm. **B**. Schematic model of M13 passing the inner membrane (IM) and outer membrane (OM) of the host envelope.

The rate of phage production is constant with about six particles released per cell per minute,[162] but because the particles are secreted, no host lysis occurs, and cell division continues, albeit at a reduced rate. Phage production ceases, however, as the cells enter the stationary phase. During an infection, a single cell can produce about 1000 phage virions in 2 hours.

M13 has been of considerable biotechnical use, first for DNA sequencing and later in "phage display."[193] Phage-display technology uses short peptides inserted into gp3 that are exposed on the surface of the phage and can be used as affinity probes. If a random sequence encoding the peptide is inserted into gene *3*, the affinity of the best binding phages for a ligand can be selected. Applications in nanotechnology, in surface chemistry, and for medical and material science have shown the broad utility of this phage display technology.

The filamentous phage CTXφ phage of *Vibrio cholerae* is of considerable interest as it encodes the cholera toxin genes.[210] CTXφ phage encodes homologs of all M13 genes, except gene *4*, which is replaced by the toxin genes *ctxA* and *ctxB*. Consequently, CTXφ phage uses the secretin EpsD in the outer membrane of *V cholera* for traversing the outer membrane. To infect new host cells CTXφ phage uses a type IV pilus, the toxin co-regulated pilus (TCP), as a receptor. The pilus genes are localized in a pathogenicity island, which resembles elements of another filamentous prophage, VPI phage.[105] CTXφ integrates into the chromosome of its host and CTXφ expression is regulated by the phage repressor, RstR. A second integrated filamentous satellite phage, called RS1, is involved in inducing CTXφ replication by binding of its antirepressor RstC to RtsR.[58] The pathogenicity of *V cholerae* is thus the consequence of a complex interplay between different filamentous phages.

Leviviridae: +ssRNA

The *Leviviridae* are among the smallest of the known phages and the only ones with ssRNA in their virions.[209] The virion RNA is of the positive

(ie, mRNA) sense and encodes four genes, with functions that are described later. There are two slightly different varieties of these phages that have been well studied, one typified by phages MS2, R17, and others, with a genome length of ~3500 bases, and a second typified by phage Qβ, with a genome length of ~4200 bases. These best-studied examples of this family of phages infect *E coli*, but similar phages have been identified that infect a variety of other hosts, including Gram-positive hosts, and virions of this family appear to be quite abundant in the environment.

The virion is a T = 3 icosahedral structure with 180 molecules of the "coat" protein and one copy of the "maturation" protein. The RNA is inside, and although it does not have the icosahedral symmetry of the protein capsid, a significant amount of RNA interacting with coat protein can be visualized in icosahedrally averaged cryo-EM structures of virions.[111] This indicates that there is significant order to the RNA within the virion. The coat protein has a fold that has not been seen in any other viruses, consisting of a β-sheet and a large α-helix. The subunits associate as dimers in which the α-helices are passed across the dimer twofold axis to link the two subunits by their α-helical "arms." The dimer is the functional unit that assembles into the capsid.

Infection starts when the virion binds, through its maturation protein, to the side of the F pilus. The maturation protein becomes proteolytically cleaved and the RNA is released into an RNase-sensitive form, which transits to the cytoplasm of the cell. How this last step is accomplished is not clear, but it may involve retraction of the pilus. The maturation protein may have a role in guiding the RNA into the cytoplasm, but once it is there the RNA alone is sufficient to initiate the infectious cycle, as demonstrated by the fact that naked RNA can be electroporated into the cell to successfully initiate infection. The virion RNA and copies of it that are made subsequently serve as the mRNA for the virus. No subgenomic mRNA is made, yet there is substantial temporal and quantitative regulation of expression from the four genes. This is accomplished by a complex interaction of the extensive and dynamic secondary structure of the RNA with ribosomes and with the replicase.

The genes are arranged on the RNA in the order 5′–[maturation]–[coat]–[replicase]–3′. In MS2-type phages a fourth gene, the lysis gene, overlaps both the end of the coat gene and the beginning of the replicase gene.[73] When the RNA first enters the cell, its secondary structure allows productive binding of ribosomes only at the start of the coat gene. As ribosomes proceed through the coat gene they disrupt RNA secondary structure that is sequestering the start of the replicase gene, allowing other ribosomes to make replicase protein with delayed kinetics relative to coat. The phage-encoded replicase protein joins with three host proteins—translation factors EF-Tu and EF-Ts, and ribosomal protein S1—to constitute the four-subunit polymerase complex that copies the RNA. Replication is a conceptually simple synthesis of a (−) strand from a (+) strand template followed by synthesis of a (+) strand from a (−) strand template. Synthesis of (+) strands is more efficient than synthesis of (−) strands, in part because the polymerase has to compete with ribosomes for access to the (+) strands through a mechanism that prevents polymerase from synthesizing RNA in the 3′-5′ direction on the (+) strand template at the same time that ribosomes are synthesizing protein in the 5′-3′ direction. The translational start site for the maturation protein is always sequestered from ribosomes except for a brief period after that part of the mRNA is synthesized, before the secondary structure that hides the ribosome binding site forms. There is time for only about one ribosome to get on the mRNA before the secondary structure forms and the site is obscured; this means there is roughly one maturation protein made for every (+) strand RNA synthesized—just the ratio that is found in virions.

Progeny virions are assembled from 90 dimers of the coat protein, one molecule of the maturation protein, and one (+) strand RNA. Neither the coat protein nor the RNA will achieve their assembled configuration in the absence of the other. It has been shown that protein and RNA each interact with the other in ways that influence its conformation and assembly capabilities, in an intricate *pas de quatre-vingt-douze* leading to a mature infectious virion.[15] In phage MS2, the protein responsible for lysis is encoded by a gene that starts within the coding sequence of the coat gene, in a different reading frame. Ribosomes do not directly enter the start of the lysis gene; rather, the ribosomes that terminate at the coat gene termination codon remain associated with the mRNA, and a small fraction of them drift back by several nucleotides and initiate synthesis of the lysis protein.[225] Cell lysis occurs when the lysis protein has accumulated to a sufficient level, but the mechanism of lysis is unknown. In phage Qβ the genes are organized slightly differently and there is no explicit lysis gene. Rather, lysis is caused by a portion of the maturation protein. In this case it has been shown that lysis is caused by inhibition of an enzyme in the cell wall synthesis pathway, MurA.[18] This is similar to what is described earlier for the φX174 E (lysis) protein, except that it targets a different step in the cell wall synthesis pathway.

At the time of the early studies of these phages, their virion RNA was the only prokaryotic mRNA available in pure, homogeneous form. As a result, it allowed the first view of what a translation start site looks like, as well as showing that some parts of an mRNA might not encode protein. In the time since, these viruses have provided extremely fruitful models for understanding how the structure and structural dynamics of RNA can influence and regulate biological processes of general importance, such as gene expression and virion assembly.

Cystoviridae: dsRNA, Segmented

Cystoviruses are unique phages that have a segmented genome composed of dsRNA and an envelope enclosing a viral nucleocapsid composed of two concentric protein shells and the genome.[12] The viral envelope fuses with the outer membrane of the Gram-negative host bacterium after receptor binding. The type member of the family *Cystoviridae* is *Pseudomonas* phage φ6, but other similar viruses have been isolated, all infecting Gram-negative hosts, mainly in the *Pseudomonas* genus. The genome consists of three linear dsRNA molecules, 2948, 4063, and 6374 bp in size, that encode 11 viral structural proteins and two nonstructural ones. The genes are clustered on the three genome segments according to their functions.[166] During the replication cycle the genome segments are converted into full-length polycistronic transcripts, each encoding four to five viral proteins. Noncoding regions at the 3′ and 5′ termini of the ssRNA molecules, flanking the internally located coding sequences, are ~370 and 310 nucleotides long, respectively. The sequence at the 5′ end displays only limited homology (17 nucleotides identity) between the segments, while the untranslated regions at the 3′ end are more similar to one another.[144]

The innermost protein shell in the virion, the polymerase complex, is composed of four protein subunits. The major capsid protein P1 is arranged on an icosahedral T = 1 lattice so that a dimer of P1 occupies the asymmetric unit. Such capsid organization is also characteristic for the dsRNA viruses infecting eukaryotic hosts.[165] Hexamers of the packaging nucleoside triphosphatase (NTPase) occupy the fivefold symmetry positions, exterior to the P1 shell. The assembly co-factor P7 and the RNA-dependent RNA polymerase P2 are located within the interior of the P1 shell. The first detectable assembly intermediate formed during phage φ6 infection is an empty polymerase complex, procapsid.[166] The three genome segments are packaged into these capsids in single-stranded form. RNA encapsidation is dependent on the segment-specific packaging signals at the 5′ termini of the viral ssRNA molecules and the activity of the packaging NTPase; it proceeds in an orderly fashion from the smallest to the largest genome segment.[144] The encapsidated ssRNA molecules are replicated within the polymerase complex into dsRNA form by the P2 polymerase utilizing a *de novo* initiation mechanism.[208] Efficient replication is dependent on 3′ terminal replication signals. Following replication the polymerase produces new single-stranded mRNA molecules from these dsRNA segments using a semiconservative strand displacement mechanism. During the encapsidation of the dsRNA genome, the polymerase complex particle undergoes substantial

conformation changes expanding the internal volume and making the particles compatible for nucleocapsid surface shell assembly.[166] The nucleocapsid surface shell follows T = 13 icosahedral symmetry and is made mainly of protein P8, which is essential during the virion entry to assist the polymerase complex particles through the host plasma membrane. The viral envelope containing four phage-encoded integral membrane proteins is derived from the host plasma membrane in a process that is dependent on the viral nonstructural protein P12. As a last step of the virion maturation, receptor-binding spike protein associates with the fusogenic protein embedded in the envelope.[166] The progeny virions are released from the infected cell by phage-induced host cell lysis.

Corticoviridae: dsDNA, Circular

Bacteriophage PM2 is the sole described member, and therefore the type organism, of the family *Corticoviridae*.[10] The virion is icosahedral, with triangulation number 21. Its coat protein has the double β-barrel fold similar to that found in PRD1 (*Tectiviridae*). Consequently, also the virion architecture resembles that of PRD1 with an internal membrane.[3] The genome is represented in a tightly supercoiled circular dsDNA molecule of 10 079 bp, inside the membrane.[133] Ten phage-encoded proteins have been identified as components of the virion: one making up the capsid shell and one forming spikes on the vertices of the capsid, and most of the others are membrane proteins. The lipids of the virion membrane are derived from the plasma membrane of the host cell, but curiously the relative amounts of the different lipids are significantly different from the average lipid composition of the host.[120]

The host of PM2 is a marine bacterium of the genus *Pseudoaltermonas*. Infection begins when the virion binds to a receptor on the cell surface leading to the release of the major coat protein and most possibly the virion membrane fuses with the host one.[108] Twenty-one protein-coding genes are inferred from the genome sequence, and the encoded proteins have been identified for 15 of them. The genes are organized into three operons. The *immediate early* operon has just three genes, is transcribed in the opposite direction to transcription of the other genes, and has sequence similarity to a region of a plasmid found in some *Pseudoaltermonas* strains. The *early* operon encodes an apparent replication initiation protein and two transcription factors necessary for transcription of the *late* operon, which encodes the virion structural components. DNA replication is thought to occur by the rolling circle mechanism, based on EM views of replication intermediates, and this interpretation is bolstered by the similarity of the replication initiation protein to the corresponding protein of phages such as φX174 (*Microviridae*), which is known to replicate its DNA by this mechanism. The mechanism of virion assembly is not well understood, but structures that appear to be membrane-enclosed DNA are seen associated with the cell membrane late in infection, and it is surmised that these are assembly intermediates around which the protein capsid assembles. In laboratory growth in rich medium, about 300 progeny virions/cell are released after a 60- to 70-minute life cycle. The particles exit from the host utilizing a novel mechanism.[114] There is a new type of holinlike protein to permeabilize the plasma membrane, and PM2 seems to use a host-derived lytic enzyme to digest the peptidoglycan. There is also a viral protein that disrupts the outer membrane of the host, a function not described previously in phage release.

PM2 was the first bacteriophage shown to have a lipid membrane as part of the virion. It is noteworthy that PM2 was isolated in the mid-1960s, yet no other examples of the family have been reported in the more than 40 years since. Very recently PM2-like sequences, which may be prophages, have been detected in the genomes of many marine bacteria.[113] This may mean that they are not that rare in the environment, and perhaps more plausibly it could mean that methods usually employed for isolating new phages fail to capture this group efficiently. This raises the question of how many novel groups of phages still await discovery.

Plasmaviridae: Circular dsDNA

The *Plasmaviridae* are a small family of phages in which the few known examples infect members of the bacterial genus *Acholeplasma*, a member of the mycoplasma. Most of the work on the group has been done with phage L2. The genome of L2 is found in the virion as an 11 965-bp circular, supercoiled dsDNA molecule.[132] Fifteen protein-coding genes have been deduced from the sequence, including at least four that encode components of the virion and one that encodes an integrase. The virions are small, quasispherical, somewhat pleomorphic structures, consisting of a membrane envelope surrounding the DNA. The locations of the viral proteins in the structure are not known.

Phage L2 has an unusual life cycle in which, instead of making a decision between lytic and lysogenic modes of growth, it does both every time it infects. That is, the first part of the life cycle is productive, producing virions that leave the infected cell by budding, without killing the cell. This is followed by the lysogenic phase in which virion production ceases and the phage DNA integrates into the host chromosome by a site-specific recombination event, apparently catalyzed by the phage integrase. Prophage-containing cells are immune to infection by homologous but not heterologous phages of the family, and lytic growth of the prophage can be induced by UV or mitomycin treatment. These observations are suggestive of a phage-encoded repressor analogous to those of phages like λ.

Evolution of Phages

Genome Mosaicism

How phages evolve has been a long-term interest in the field, and the scope and specificity of what can be learned have expanded greatly with the availability of high-throughput DNA sequencing. To somewhat oversimplify, phages have been used in two ways in evolutionary studies. In the first, phages are put into a controlled environment in the laboratory and followed by genome sequencing through various selections, competitions, population bottlenecks, and so forth. This approach has allowed rigorous testing of a number of different aspects of evolutionary theory and made it possible to elucidate specific pathways of adaptation, typically at the single nucleotide level, and in this way to illustrate individual evolutionary events that must take place in natural phage evolution. This work has been very valuable but has not necessarily represented most of the enormous and chaotic complexity of the natural environments where phages evolve nor the extraordinarily long times over which phage evolution is thought to have occurred.[27,155] The goal of understanding the mechanisms of evolution as actually practiced by phages in the wild has been more closely approached by isolating phages from the environment and sequencing and comparing their genomes.[85] This approach has the limitation that the sampling of the population is extremely sparse and almost certainly biased, but it has nevertheless yielded a wealth of information about the structures of the genomes produced by phage evolution, and this has allowed inferences about the mechanisms by which they got there. In thinking about phage evolution it is helpful to keep in mind the numbers cited in the first paragraph of this chapter. For example, there are ~10^{24} infections per second on a global scale for the tailed phages alone (see earlier), and each of those infections is an opportunity for genetic rearrangement. As we describe later, there is reason to believe that this has been going on for something

like the past 10^{17} seconds. Most of the discussion given later relates to the tailed phages, for which the most information is available; there is also some comparable work available for the *Microviridae*, which has produced similar but not identical conclusions about mechanisms in this different group of phages.

If the genomes of a closely related group of tailed phages are compared, they are seen to be genetic mosaics with respect to each other. That is, the sequences of two genomes may be nearly identical over the length of a few genes and then abruptly change so that they match less well or not at all.[38,94] These sites are the "fossils" of nonhomologous recombination (ie, recombination between two DNA sequences that are different from each other) in the ancestry of one of the phages, creating a "novel joint" in the genome such that two sequences are placed adjacent to each other that were not adjacent in either parental sequence.[158] This is the mechanism by which these phages are thought to achieve horizontal exchange of DNA sequences, and horizontal exchange is a central feature of the evolution of these phages. The locations of the novel joints are informative; in most cases they have recombined between genes or at gene boundaries. This was initially thought to suggest that recombination events occurred preferentially at gene boundaries.[199] However, closer examination of the data argues that nonhomologous recombination occurs promiscuously across the entire genome without site specificity, but that the vast majority of recombinants are eliminated by natural selection. The recombinants that survive are those that do not disrupt function, and most of these are at gene boundaries, or in other words, not within protein-coding regions. The exceptions that prove the rule are novel joints that fall within protein-coding regions but at positions corresponding to functional domains of the protein. The tyrosine integrases, tail fibers and tailspikes, and DNA packaging proteins provide good examples of this. Opportunities for recombination between different phage genomes arise when a cell is infected simultaneously by two phages; however, a more frequent opportunity for recombination occurs when the infected cell carries one or more prophages, as is likely true for a great majority of cells.[36]

Homologous recombination (ie, between identical sequences) also certainly has a significant role in the reassortment of genes among genomes in that homologous recombination between identical sequences on different genomes can reassort the novel joints and dissimilar genes that flank the identical sequences. These identical sequences may be identical protein-coding sequences. In addition, there are scattered examples of identical stretches of sequence shared between intergenic regions, and more generally, intergenic regions may share more than random sequence similarity due to shared occurrence of transcription and translation signals, and this may bias recombination toward these locations.[48]

There are apparent constraints beyond those described earlier on where recombination between genomes can occur (and survive natural selection to be seen by us). Thus, in the smaller tailed phages like λ and its relatives, there is little or no mosaicism seen within the group of procapsid assembly genes and usually also little mosaicism within the group of tail tip assembly genes. Similarly, genes for DNA binding proteins, for example, the λ *cI* repressor gene, "travel through evolution" together with the DNA sites their encoded protein binds. Such cases are thought to be explained by shared function of the genes that show this behavior. Thus, the proteins encoded by the head genes of a phage like λ must each interact intimately with the other head proteins during head assembly and in the assembled structure, and they have co-evolved over time to maintain productive interactions among the group. Substitution of a homologous gene or genes from a phage that had followed a different path of mutation and co-evolution would in almost all cases be expected to produce a nonfunctional set of head genes and be counterselected, even though all the genes would be functional in their home genomes. A notable exception to such co-evolution is the parts of tail fiber and tailspike genes that bind bacterial receptors. They appear to have been horizontally exchanged many times among even very different phages. For example, there are members of the *Podoviridae*, *Siphoviridae*, and *Myoviridae* that, in spite of having very different life cycles, have nearly identical tailspikes that utilize the same host receptors, and it is unlikely these arose through convergent evolution. It seems that sometimes when a phage manages to obtain a new host specificity in this way, it can have an advantage, because it can enter an evolutionarily naive cell that is not prepared to defend itself against a phage that it has not "seen" before.

Common Ancestry of Archaeal and Eukaryal Viruses With Phages

Homology (shared ancestry) among viral genes can be inferred from similarity of their encoded amino acid sequences, as is done routinely, for example, for genes of mammalian genomes. However, this approach has turned out to be quite limited for viral sequences because the sequences of viral proteins that are thought on other grounds to be homologs have often diverged to the point that they can no longer be recognized as similar. This has meant that, while it is possible to construct phylogenetic trees based on protein sequences from closely related viruses, there is little hope of inferring the deep evolutionary history of viral proteins or their associated viruses based on sequence comparisons alone. Happily, this situation has changed somewhat with the advent of structural information about viral proteins—mostly virion structural proteins—in recent years.

The capsid *hexons* of bacteriophage PRD1 (*Tectiviridae*) are homotrimers of a protein subunit that has two domains, each of which is a β-barrel of the *jelly-roll* type.[11] Each of the jelly-rolls occupies a position in the capsid lattice that would be occupied by a single capsid protein subunit in a classical Caspar-Klug structure. The high-resolution structure of the PRD1 hexon was a surprise when it was first determined because the aforementioned description applies equally well to the hexons of adenovirus.[16] The similarity extends to the protein folds of the two hexon proteins, and this has led to the inference that these two proteins may share common ancestry, despite the fact that their amino acid sequences no longer share any detectable similarity. The same double jelly-roll fold has been found subsequently in eukaryal viruses PBCV1 (*Phycodnaviridae*) and Mimivirus (*Mimiviridae*); Sulfolobus turreted icosahedral virus (STIV; *Rudiviridae*), which infects the hyperthermophilic archaeon *Sulfolobus;* and bacteriophage PM2, arguing that this diverse and sequence-dissimilar group of capsid proteins also shares in the PRD1 and adenovirus ancestry.

The tailed phages (*Caudovirales*) have a capsid protein with a very different fold, typified by that of *E coli* phage HK97.[217] Evidence to date indicates that this fold is shared across most of the tailed phages, including those that infect archaeal hosts, often in the absence of detectable sequence similarity. It also appears that this same "HK97 fold" makes up the shell-forming domain of the herpesvirus capsid protein, arguing for a common ancestry with the tailed phage capsid proteins for the herpesvirus protein as well.[102] The case for common ancestry between tailed phage and herpesvirus capsid proteins is bolstered by shared mechanisms of capsid assembly and DNA packaging, including faint but convincing sequence similarity between the DNA-packaging proteins of phage T4 and herpes simplex virus. A third capsid protein fold reaching across host domains includes phage φ6 (*Cystoviridae*) and the reoviruses of plant and animal hosts. These viruses have double protein shells; the inner shell has the noncanonical T = 2 structure with a novel protein fold and the outer shell is a T = 13 shell of single jelly-rolls.[165] Together these observations lead to the view that these viruses define at least three viral lineages, or perhaps more properly three viral capsid lineages, whose hosts span all three domains of cellular life (Fig. 21.14). A simple (but not yet firmly established) interpretation is that viruses with the basic capsid characteristics of these contemporary viral lineages had already evolved by the time of the last common cellular ancestor of the cellular domains of life. There are of course viruses with icosahedral capsids but without any of the capsid protein folds of these three lineages,

for example, the *Leviviridae* (small ssRNA genomes) among phages. The capsid protein folds associated with these other viruses are thus far associated only with hosts within one cellular domain, and it will be interesting to see how that situation changes as more viruses are identified and characterized. There are also other examples of virion protein structural similarities among very different virus types. For example, fibers that protrude from the icosahedral vertices of adenoviruses and tectiviruses, as well as fibers that extend from some phage tails, have knobs at the distal tips. The polypeptide folds of these three knobs are sufficiently similar to suggest a possible common ancestry.[21]

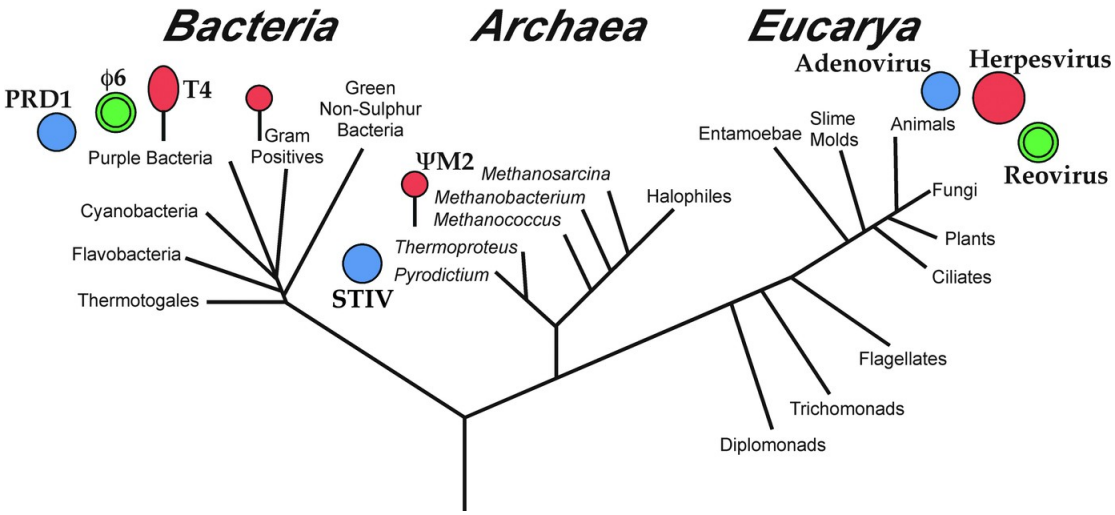

FIGURE 21.14 **Deep evolutionary connections among viruses**. The tree represents the tree of cellular life. The colored symbols represent the indicated viruses, placed near their cellular hosts. Shared color indicates shared "viral lineage," as deduced from shared structural features of the virions.

Phage Parts in other Contexts

Structures are occasionally found in bacterial or archaeal cells that bear a structural resemblance to virions—or parts of virions—of the tailed phages. For the examples given here, examination of the polypeptide folds of the components of the structures, and in most cases, comparisons of their amino acid sequences, makes a strong case for common ancestry shared by the virions of tailed phages and these "cellular" structures.

Pyocins

The pyocins are a class of high-molecular-weight bacteriocins encoded in the genomes of some *Pseudomonas* strains.[142] They have the morphology of phage tails, either contractile or not, and they kill sensitive cells after they are released from the cell that encodes them, apparently by making holes in the cell envelope and causing collapse of the cellular membrane potential. The genes encoding the pyocins have close sequence similarity to the tail gene operons of well-studied phages—*E coli* phages P2 (contractile tail) and λ (noncontractile tail), respectively—in the well-characterized cases of the R2 and F2 pyocins of *P aeruginosa*.[142] The high sequence similarity between the respective pyocin and phage tail operons bespeaks an evolutionarily recent common ancestry.

Gene Transfer Agents

Gene transfer agents, or GTAs, were discovered as a generalized transducing activity in cultures of *Rhodobacter capsulatus*.[124] They were subsequently shown to be small particles with the morphology of a tailed phage that package short (4.5-kbp) pieces of DNA derived from apparently random locations around the *R capsulatus* genome. GTA-like particles with similar properties have been identified in several other bacterial and archaeal species, but the *R capsulatus* GTAs are the best characterized. The GTA particles are encoded by a ~14-kb-long operon of genes in the *R capsulatus* genome. In both gene organization and sequence similarity, the genes of this operon strongly resemble the head and tail genes of a tailed phage with a noncontractile tail. The GTA operon bears some resemblance to a prophage, and it may well have derived from one, but it is not a conventional prophage that has suffered deletion of its nonvirion assembly genes for at least two reasons. First, the heads of the particles can only package the equivalent of about one-quarter of the genes that encode the particle. Second, expression of the GTA genes is under the control of a cellular two-component system that senses conditions outside the cell. It appears that the cell may have co-opted the GTA genes to serve its own purpose. What that purpose might be is not known, but a plausible possibility is that the GTA particles are genetic repair kits, sent from one cell to other cells in the population, to save the population from the ravages of Müller's ratchet.

Encapsulins

The encapsulins are a recently described group of subcellular shells found in both bacteria and archaea.[52] In cases where their function has been inferred, they enclose and sequester biochemical reactions that produce potentially harmful reactive intermediates. The shells are icosahedral structures, T = 1 or T = 3, made of protein subunits that bear clear homology to the phage HK97 family of capsid proteins, based both on close structural similarity and on a low but unequivocal level of amino acid sequence similarity. The genes encoding the encapsulin subunits are not associated with any other phagelike genes in the cellular genome.

Type VI Secretion Systems

Recent structural studies of the proteins of the needles of the *Pseudomonas* and *Edwardsiella* type VI secretion systems (T6SS) show that the

major subunits of the needles have the same polypeptide fold as the tail tube protein of phage T4 (gp19), despite a lack of recognizable shared sequence.[101] This is taken to suggest a common ancestry for the two proteins and, by extension, possible common ancestry for the phage tail and the T6SS apparatus.[14] A parallel study with the phage λ tail tube subunit, gpV, shows that it shares the gp19 fold, arguing that the common ancestry inferred for the tail tube of a contractile tailed phage and the T6SS needle extends to the noncontractile phage tails as well.[160] Recent structural studies have identified a long contractile structure, resembling an exceptionally long contractile phage tail, in cells indulging in type VI secretion[122] (Fig. 21.15). This structure assembles in the bacterial cell as it is preparing for secretion and disassembles after. It appears to drive the T6SS needle into the target cell by contraction of the sheath, just as a contractile phage tail drives the tail tube through the envelope of a target bacterium.[35]

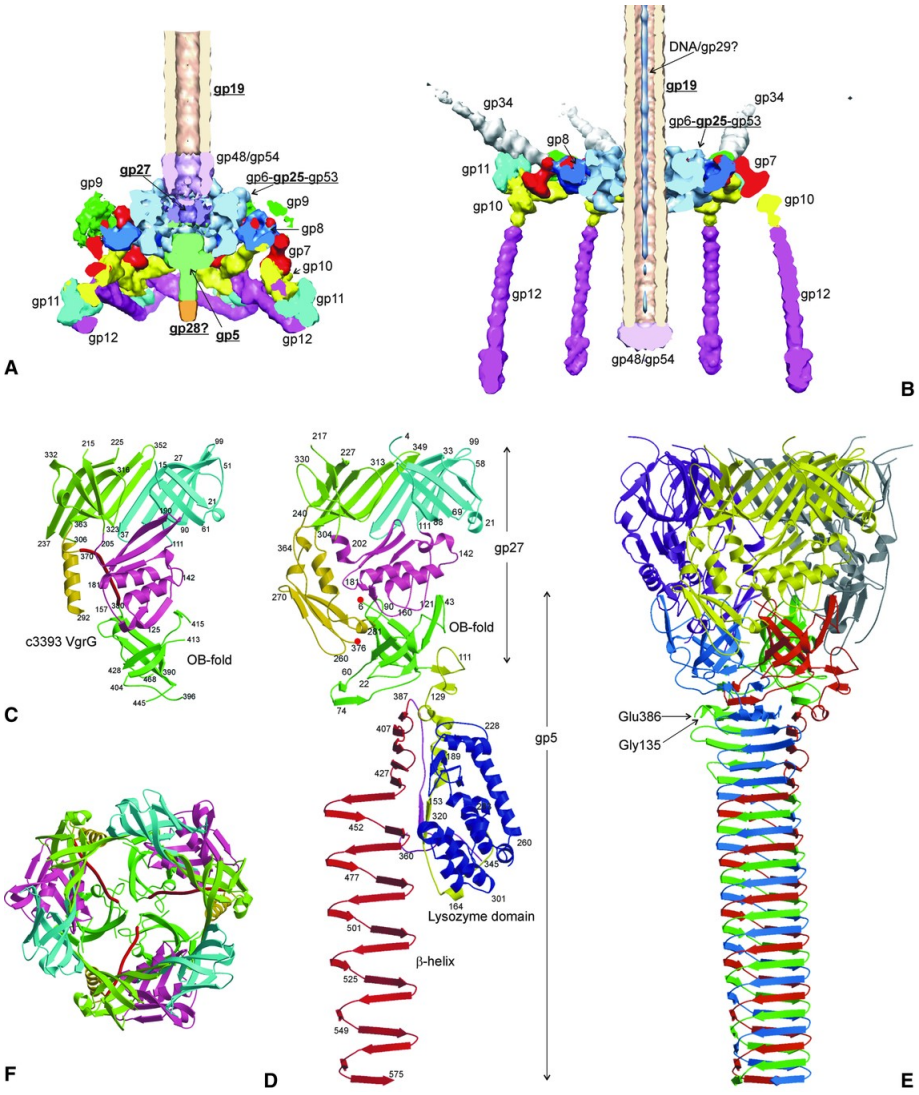

FIGURE 21.15 **Structure of the bacteriophage T4 baseplate and comparison of the *Escherichia coli* CFT073 c3393 VgrG with its T4 homologs, gp5 and gp27**. Cryoelectron microscopy (cryo-EM) reconstructions of the T4 baseplate before **(A)** and after **(B)** attachment to the host cell. Component proteins are labeled with their respective gene numbers. The T6SS protein homologs are highlighted in bold and underlined. **C**. The crystal structure of the c3393 VgrG. Different domains are colored in distinct colors. The gp27 tube domains are colored cyan and light green. The fragment of the polypeptide chain connecting the gp27 and gp5 modules is shown as a thick red tube. **D**. The structures of gp5 and gp27 monomers extracted from the (gp5)3-(gp27)3 complex. The terminal ends of the gp5 and gp27 polypeptide chains, which become fused in the VgrG structure, are highlighted with *red dots*. **E**. A model of the prototypical VgrG is created from the entire (gp5)3-(gp27)3 complex by removing the lysozyme domain. **F**. End-on view of the crystal structure of the c3393 VgrG trimer. (Reprinted with permission from Leiman PG, Basler M, Ramagopal UA, et al. Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. *Proc Natl Acad Sci U S A*. 2009;106(11):4154-4159.)

In all of these cases, there seems little doubt that the structural proteins of phages and the corresponding proteins of phagelike structures in bacterial and archaeal cells share common ancestry. A simple and generally accepted explanation for this relationship is that a group of phage genes has been captured by the host cell, possibly from a resident prophage, and placed under a regulatory scheme that benefits the host. In this view, such acquisition of phage virion structural genes is simply an example of the more general phenomenon of cells acquiring “phage” genes and converting them over time into “cellular” genes. The difference in the case of the structural genes is that they are more easily recognized as being of phage origin, even after mutational drift has eliminated any detectable sequence similarity. However, despite the plausibility of tailed phages as the progenitors of the cellular structures enumerated earlier, there is no formal proof that the arrow might not go in the other direction—that is, that T6SS needles, for example, might be the ancestors of phage tails!

Host Interactions

The essence of being a virus is interacting with the host, and these interactions influence both virus and host. We will consider some examples of interactions between phages and bacteria in three somewhat artificial and overlapping categories.

Parasitic Interactions

Phages hijack their hosts' biochemical machinery, usually to the detriment of the host, and as in other areas of virology, understanding how these interactions work often tells as much about the cell as about the virus. The ways these interactions are carried out are as varied as the phages doing them; we give examples here of some of the better-studied cases.

DNA Replication

Some phages such as phage T4 encode all the proteins needed to replicate the phage genome. These form a complex that replicates the phage DNA with a preference for the modified cytosine nucleoside that T4 uses in place of cytosine. At the same time other phage-encoded enzymes are degrading the host DNA and recycling the nucleotides (in conjunction with some host nucleotide metabolism enzymes) for use by the phage replication machine. *Bacillus* tailed phage SPO1 has at least nine genes required for phage DNA replication but appears to be lacking some of the complete set of replication genes that a phage like T4 has.[194] SPO1 uses hydroxymethyl uracil in its DNA in place of thymine, and the phage encodes several enzymes with roles in depleting thymine pools and synthesizing hmU, and the phage-encoded DNA polymerase has an extra domain that is proposed to help it recognize the hmU-containing template. Many phages use the complete host replication machinery, synthesizing only the proteins needed to recruit the replication complex to the phage DNA template. An example is phage λ, which makes two replication-specific proteins, one of which (gpO) binds the phage replication origin and one (gpP) that recruits the host replication helicase, and through it the rest of the host replication complex, to the phage origin.[203] Unlike T4, λ does not degrade the host DNA, presumably because doing so would preclude the possibility of forming a lysogen. Phage φX174 also uses the host replication machinery. However, unlike the host and unlike most other phages, it uses the host transcription RNA polymerase to lay down the RNA primers at the start of new DNA chains, rather than primase, the specialized RNA polymerase that usually carries out the priming function. For technical reasons, this peculiarity of φX174 facilitated the original discovery that RNA primers have a role in DNA replication. The ssRNA phage MS2 encodes a replicase protein, but to replicate the RNA it co-opts the activities of three cellular proteins (EF-Tu, EF-Ts, S1) that normally interact with RNA but to very different ends.

Transcription

Most DNA phages use the host RNA polymerase for some or all of their transcription. They often achieve temporal separation of different classes of transcripts by successive modifications of the host polymerase. For phage λ the transition from *immediate early* to *early* transcription is mediated by the immediate early antitermination protein gpN, which interacts with the nascent RNA transcript and the RNA polymerase to render the polymerase able to read through termination signals.[175] The transition from *early* to *late* transcription is mediated by another antitermination protein gpQ, acting by a different mechanism. Phage T4 makes covalent modifications to the host polymerase early in infection and adds a phage-specific sigma factor to the polymerase to effect the transition to late transcription.[96] Phage T7 transcribes its early genes with the host RNA polymerase, but one of these genes encodes the phage RNA polymerase, which is responsible for subsequent transcription of phage genes. Another early gene of T7 encodes a kinase that inactivates host transcription by phosphorylation of the host RNA polymerase.[141] A phage that flouts all the normal conventions for organizing transcription is *E coli* phage N4. N4 packages a few copies of a *virion RNA polymerase* in the virion with the DNA, and they are injected into the cell along with the DNA.[60] The virion RNA polymerase transcribes the early genes, some of which encode a second (phage-encoded) RNA polymerase that transcribes the middle genes. The middle genes include genes supporting phage DNA replication and also an ssDNA binding protein, which interacts with the late promoters in such a way that they can be read by the unmodified *E coli* $\sigma(^{70})$-RNA polymerase!

Translation

All phages, like all viruses, use the host translation apparatus to make their proteins. For the most part the phages appear to use it in unaltered form, though they do make use of some of the "special abilities" that ribosomes have beyond the textbook descriptions of translation. These include programmed translational frameshifts (many phages), a 50-nt ribosomal "hop" (phage T4), a few examples of regulation of gene expression at the level of translation (phage T4), and the extensive examples of regulation of translation described earlier in the section on the *Leviviridae* (the ssRNA phages).[98,223] For many tailed phages, particularly (but not exclusively) those with larger genomes, there is suggestive evidence that they also make specific alterations to the host translation apparatus. The most frequently encountered support for this assertion is in the form of phage-encoded tRNAs. Although the majority of characterized phages do not encode any tRNAs, there are many that do. The numbers of apparent tRNAs per genome in this group range from 1 to >30, with their genes usually arranged in one or more clusters on the genome. Some of these putative tRNAs give high scores in tRNA search programs trained on cellular tRNAs and some do not, and in only a few cases have they been tested and shown that they can actually function as tRNAs. It has been suggested that the purpose of the phage-encoded tRNAs is to adjust the tRNA pools in the infected cell to better serve the needs of phage translation, but they may have other roles that are not yet identified. Several phages have been found to carry a copy of a transfer-messenger RNA (tmRNA), the RNA found in prokaryotes with features of both tRNA and mRNA and a role in releasing stalled ribosomes from mRNA. Finally, phage G of *Bacillus megaterium*, the phage with the largest genome known at 498 kb, has one aminoacyl tRNA synthetase.[90] This last feature is reminiscent of the very large Mimiviruses, which also carry aminoacyl tRNA synthetases for which the biological role also is not known.[50] These observations taken together make a strong case that some phages modify the host translation machinery, but there is as yet little specific information about how or to what end they might actually do this.

Other Cellular Machinery

Phages rely on their hosts for energy metabolism. They also typically use the cellular biosynthetic machinery to make precursors for macromolecular synthesis, but a number of phages encode enzymes with roles in nucleotide metabolism. When these are present, they typically act in conjunction with cellular enzymes of nucleotide metabolism. Two of the most easily recognized and frequently found nucleotide metabolism genes in phages encode ribonucleotide reductase and thymidylate synthetase. To date, there is no evidence of phages participating in amino acid biosynthesis, nor of phages incorporating noncanonical amino acids into their proteins. For phages with lipid membranes in their

virions, the lipids appear in all cases to be derived from the cellular membrane.[3]

Phages also make use of host chaperone proteins, and in fact some of the most important of these were discovered because of their roles in the life cycles of phages λ and T4. The *E coli* DnaK and DnaJ proteins (also known as Hsp70 and Hsp40) were found to be required for λ DNA replication, where they partially disassemble the replication initiation complex and allow the replication machinery to leave the origin and begin synthesis. GroEL and GroES (Cpn60 and Cpn10) are required for accurate assembly of phage λ heads, now understood as a consequence of their role in mediating correct folding of some of the head proteins. GroEL is also required for phage T4 head assembly because of its role in folding the major capsid protein gp23. The gp23 subunit is apparently slightly too big to fit into the GroE folding cavity with the GroES "lid" in place, and T4 encodes its own homolog of GroES, gp31, which leaves enough more space in the cavity for successful folding of gp23. We note that these two chaperones, now known as the hsp70 (DnaK) and hsp60 (groE) systems and known to be very important and present in virtually all organisms, were originally discovered in work designed to understand the molecular nature of these phage infections.

Phages interact in significant ways with cellular structures, particularly the cell wall and membranes, during cell entry and exit. The *Leviviridae* (ssRNA, icosahedral) and *Inoviridae* (ssDNA, filamentous) of *E coli* bind to the sides and tip, respectively, of the F pilus, and they are thought to be brought to the surface of the cell for further interactions by retraction of the pilus. The tailed phages Cb13 and CbK of *Caulobacter crescentus* have helical tail fibers that wrap around the shaft of the bacterial flagellum.[82] This makes an interaction that is apparently able to slide along the helical flagellum, and when the flagellum rotates in the appropriate direction the phage is screwed down to the surface of the cell where it interacts with a cell surface receptor.

Antagonistic Interactions

Phages and their bacterial hosts engage in ongoing "biological warfare" in which mutations arise in the bacterium that block successful lytic growth by the phage, which in turn creates a selective advantage for phages with mutations that overcome the block. The best-known examples are mutational changes that affect the binding between the virion and its receptor on the surface of the cell. In the tailed phages, a single amino acid change in the tail fiber protein can be sufficient to restore binding to a recalcitrant cellular receptor, but bigger changes occur as well. The parts of tail fiber genes that encode the receptor-binding part of the tail fiber often show evidence of recent horizontal exchange, and in some phages rapid changes of sequence, specifically in tail fibers, are programmed into the phage genome, as in the examples of phage Mu and phage BPP-1.[137,184] The biological warfare continues after the phage has successfully gotten its genomic DNA or RNA into the cell. Many examples could be given; a particularly piquant example comes from the *prrC* gene of *E coli*. The product, PrrC, is a nuclease that cuts in the anticodon loop of the cellular $tRNA^{Lys}$, inactivating it and so inhibiting protein synthesis in the cell.[207] PrrC is ordinarily held in an inhibited form, but infection by phage T4 causes its release in active form, whereupon it cleaves this tRNA. The "purpose" of the cleavage is evidently to prevent the phage from making virions that could infect other cells in the population. However, T4 encodes two enzymes, polynucleotide kinase and RNA ligase, that counter this host defense by repairing the tRNA. Relatives of T4 that lack the repair enzymes are unable to grow on *E coli* strains that carry *prrC*. A more widely distributed host defense is the CRISPR system, sometimes referred to as bacterial (and archaeal) adaptive immunity.[62] Cells with a CRISPR system have a mechanism to collect an array of short (~25-bp) DNA sequences derived from phages of past infections (which the ancestral cells evidently survived). When a phage that has an exact match to one of the sequences in the CRISPR array infects, it is inactivated by a mechanism that may have some features in common with the eukaryotic small interfering RNA (siRNA) mechanism. Phages can escape CRISPR surveillance if they acquire as little as a single nucleotide change in the sequence being recognized.

Symbiotic Interactions

When a phage enters the lysogenic cycle and becomes a prophage, most of the genes are repressed by the repressor protein. However, some phage-encoded proteins continue to be made from the repressed prophage—most obviously the repressor protein itself—and the list of other proteins expressed from an otherwise repressed prophage is growing.[25] These proteins all have the potential to cause lysogenic conversion of their host cell, that is, a change in cell phenotype as a consequence of the protein's expression. In many cases, the biological function of these lysogenic conversion genes is not known, but when it is, it can typically be construed as providing a selective benefit to the host cell in which the prophage resides. Thus, the phage λ prophage expresses the repressor and three other proteins, SieB, RexA, and RexB, each of which protects the cell from infection by certain other phages, and it also expresses two proteins, Bor and Lom, which are thought to make the lysogenic cell better able to parasitize a mammalian host.[13] Other such proteins are described later. It has been suggested that these lysogenic conversion genes do not provide a selective benefit to the phage directly but instead provide a selective benefit indirectly by providing a direct benefit to the lysogenic cell in which the prophage is being replicated. In other words, the lysogenic conversion protein is the rent the prophage pays to its host. It may also be that lysogeny provides a rather efficient pathway for the bacterium to acquire novel beneficial genetic functions. That is, as random deletions occur over time in the bacterial chromosome, any that remove one of the beneficial lysogenic conversion genes will be counterselected, whereas deletions that remove other parts of the prophage will be either neutral or selected. Over time, only the beneficial genes will remain as part of the bacterial genome.

One of the best-studied groups of lysogenic conversion genes is the group of prophage genes known to encode toxins produced by bacterial pathogens.[34] The toxins of the human diseases botulism, diphtheria, cholera, and hemolytic uremic syndrome (HUS), among others, and the animal disease ovine foot rot, are encoded by prophage genes. The Shiga-like toxin encoded by *E coli* 0157:H7, the causative agent of HUS, is of particular interest. This toxin is expressed at low levels from its own promoter, but the higher levels of toxin required to produce severe disease are only made when the prophage is induced and the toxin is produced under the control of the phage's strong late promoter.[206] Some antibiotics cause induction of this prophage, and for this reason antibiotic treatment is contraindicated for this infection. The cholera toxin case is also noteworthy in that the toxin is carried on a prophage of a filamentous ssDNA phage of the *Inoviridae* family.[59] All the other known examples involve prophages of the tailed dsDNA phages of the *Caudovirales* order.

Because studies of lysogenic conversion have been carried out by human biologists, it is not surprising that some of the best-studied examples of lysogenic conversion involve toxins of human pathogens. However, it is unquestionably true that there are prophages supplying selectively beneficial functions to their host bacteria (and host archaea) in virtually all of the ecological situations where these cells find themselves.

Phage Communities and Ecology

Every gram of soil and sediment, every milliliter of water, and the surfaces and insides of plants and animals are hosts to extensive and diverse

phage and bacterial communities. We now know that these environmental phage communities contain most of the uncharacterized genetic diversity on Earth. And because of their rapid killing of large numbers of bacteria, they strongly influence the flow of carbon, phosphorus, and other elements within the biosphere.

Metagenomic analyses of viral communities, also called viromes, have shown that 1 kg of marine sediment may contain over a million different phage genotypes (roughly equivalent to a species).[23] Similarly, there are hundreds of thousands of viral species in the ocean and soils.[72] Viromes from stool samples show that most of the genomic differences between individual humans are gut-associated phages.[173] There are even phages in our blood.[76]

Phage could potentially protect against pathogens by killing them, but phage may actually be the causes in others. For example, many bacterial pathogens are actually lysogens and their prophages carry the disease-causing genes. For example, the common mouth microbe *Streptococcus mitis* carries the prophage SM1. When induced, the phage SM1 virions carry modified tail fibers known as pblA and pblB, which "glue" *S mitis* cells together and to platelets. If this happens in the blood, say, after tooth cleaning, then the phage-platelet-microbe blob can cause endocarditis. Scarily, the phage SM1 is induced by soy sauce and other more commonly ingested substances; eat up[220]!

Bacterial communities in the environment are often controlled in a top-down manner; that is, there is more food available than the microbes use.[56,218] This occurs because they are killed off by phage and other predators like unicellular protists. The continuous predation by phage leads to "kill the winner" dynamics, where one phage kills so much of a particular bacterial strain that another strain grows up in the unoccupied niche. This means that the dominant bacterial strain within an ecosystem will be constantly changing. Recent work has shown that kill-the-winner dynamics does not change the species of bacteria; rather, it causes cycling of different strains.[177]

Our understanding of phages in the environment is in its infancy. At this point we know that there are almost unimaginable numbers of them, and they contain most of the uncharacterized genomic diversity on the planet. It is clear that to maintain the observed steady state, phages are influencing both the types and replication of bacteria, and it is also clear that there is an amazing, essentially unexplored world of phage diversity to be studied.

Prospects for Therapeutic Uses of Phages

The idea of using phages to control human bacterial infections was recognized by d'Herelle soon after their discovery around 1917. Only 30 years earlier the germ theory of disease was becoming accepted, and the rules for etiology established by Koch. A vigorous search for cures based on killing bacterial pathogens began in the late 19th century, and there were numerous false claims of success, including Koch's assertion of tuberculin as a cure for consumption.[221] Thus, the possibility of using d'Herelle's newly discovered phages therapeutically would have been a welcome and timely discovery, coming still several years prior to Fleming's penicillin (1928) or Schatz and Waksman's streptomycin (1943), and the widespread introduction of antibiotic therapies in the 1940's.

Nonetheless, the therapeutic use of bacteriophages ("phage therapy") has been a turbulent subject for the past 100 years. D'Herelle's research, together with that of George Eliava, in Tbilisi, Georgia prompted broad use of phage therapy in the former Soviet Union, although it was not adopted early in the United States and Europe, and became largely ignored with the widespread use of antibiotics.[197] Phage therapies were likely successful for at least some infections in the early days in the USSR, although the literature is sparse and mostly in Russian; double-blinded randomized clinical trials are rare or not easily accessible. After several hiatuses phages are still provided for treating infections in Tbilisi, in Poland, and elsewhere.[227] Some of the most important lessons emerging from these experiences are that "success" often depends on isolating and propagating phages on a specific pathogenic strain.

General interest in the therapeutic use of phages has cycled over the past 30 years, partly in response to the growing limitations of antibiotic control of bacterial infections balanced against the challenges in advancing phage therapeutic use.[84] Over the past 5 years, there has been more intense interest in light of the growing severity of antibiotic resistance and some successful case studies. In addition, innovations in the biotechnology community supporting intellectual property protections and promoting financial investment are fueling new approaches towards therapeutic success. Nonetheless, the future of phage therapy remains unclear, with considerable uncertainty over its broad applicability but some confidence that phage treatments have important roles to play.

Three recent examples of successful therapeutic use of phages illustrate notable advances. First is the use of phage cocktails to treat a highly antibiotic resistant disseminated infection of *Acinetobacter baumannii*.[188] The case is notable because of personalization of the phages for specificity against the particular bacterial isolate, the use of multiple cocktails to counter resistance, the likely interaction between the phages and ongoing antibiotic therapy, and safe intravenous administration. The patient was sufficiently sick and antibiotics sufficiently inept that he would have surely died without phage intervention. Enthusiasm should be tempered before concluding that phage therapies are a general treatment for *A baumannii* infections, but it suggests strongly that phages may be an important component of treatment options. Although there were no adverse reactions to the phage treatment in this case, it was essential that the level of contaminating lipopolysaccharide endotoxin be reduced to avoid severe complications following IV administration.

The second case is the use of phages to treat a highly antibiotic-resistant *Mycobacterium abscessus* infection in a pediatric cystic fibrosis patient who was immunosuppressed following a bilateral lung transplant.[61] This was the first successful use for treating any *Mycobacterium* infection with phages and has implications not only for the treatment of nontuberculous *Mycobacterium* (NTM) infections but potentially for human tuberculosis, a disease that afflicts many millions of people across the globe.[81] In this case it was also necessary to personalize the phage cocktail to the patient's isolate, and the phages were administered intravenously without adverse reactions. This case is also notable as it was the first therapeutic use of engineered phages. Most of the phages infecting *Mycobacterium* pathogens are temperate and thus unsuitable for therapy. However, tools for efficient engineering of mycobacteriophages have been developed[134,215] facilitating precise removal of the repressor and integrase genes, converting these to lytic phages with therapeutic utility.

In the third case, phages were used to treat an aortic arch prosthesis with a severe *Pseudomonas aeruginosa* infection that was unresponsive to antibiotics.[45,46] This case is notable because the phage used (OMKO1) was chosen as it binds to an outer-membrane efflux system, and loss of the efflux system contributes to phage resistance, while sensitizing the bacteria to several antibiotics. The phage also disrupted *P aeruginosa* biofilms on the implant surface. Treatment was successful, and the implant remained sterile after cessation of phage and antibiotic treatments.

These examples are among many suggesting that bacteriophage treatments have a role to play in the control of bacterial infections. What that role is remains unclear, although it will almost certainly differ for different infections and different pathogens.[1,84] If it restricted to personalized treatments of last resort it will be relatively expensive and challenging to bring to the marketplace. A broader utility—working together with antibiotics and helping to minimize the emergence of antibiotic resistance—is likely achievable for at least some infections but will require an understanding and management of phage host range and the bacterial mechanisms of phage sensitivity and resistance.

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REFERENCES

1. Abedon ST. Use of phage therapy to treat long-standing, persistent, or chronic bacterial infections. Adv Drug Deliv Rev. 2019;145:18-39.

2. Abrescia NG, Cockburn JJ, Grimes JM, et al. Insights into assembly from structural analysis of bacteriophage PRD1. Nature. 2004;432(7013):68-74.

3. Abrescia NG, Grimes JM, Kivela HM, et al. Insights into virus evolution and membrane biogenesis from the structure of the marine lipid-containing bacteriophage PM2. Mol Cell. 2008;31(5):749-761.

4. Ackermann HW, DuBow MS, Gershman M, et al. Taxonomic changes in tailed phages of enterobacteria. Arch Virol. 1997;142(7):1381-1390.

5. Adriaenssens EM, Sullivan MB, Knezevic P, et al. Taxonomy of prokaryotic viruses: 2018-2019 update from the ICTV Bacterial and Archaeal Viruses Subcommittee. Arch Virol. 2020;165(5):1253-1260.

6. Al-Shayeb B, Sachdeva R, Chen LX, et al. Clades of huge phages from across Earth's ecosystems. Nature. 2020;578(7795):425-431.

7. Anderson DL, Hickman DD, Reilly BE. Structure of Bacillus subtilis bacteriophage phi 29 and the length of phi 29 deoxyribonucleic acid. J Bacteriol. 1966;91(5):2081-2089.

8. Anderson LM, Yang H. DNA looping can enhance lysogenic CI transcription in phage lambda. Proc Natl Acad Sci U S A. 2008;105(15):5827-5832.

9. Arisaka F, Yap ML, Kanamaru S, Rossmann MG. Molecular assembly and structure of the bacteriophage T4 tail. Biophys Rev. 2016;8(4):385-396.

10. Bamford DH, Bamford JKH. Lipid-containing bacteriophage PM2, the type-organism of Corticoviridae. In: Calendar R, ed. The Bacteriophages. Oxford University Press; 2006: 171-174.

11. Bamford DH, Grimes JM, Stuart DI. What does structure tell us about virus evolution? Curr Opin Struct Biol. 2005;15(6):655-663.

12. Bamford DH, Poranen MM. Family cystoviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, eds. Virus Taxonomy. Elsevier; 2011:515-518.

13. Barondess JJ, Beckwith J. bor gene of phage lambda, involved in serum resistance, encodes a widely conserved outer membrane lipoprotein. J Bacteriol. 1995;177(5):1247-1253.

14. Basler M, Pilhofer M, Henderson GP, Jensen GJ, Mekalanos JJ. Type VI secretion requires a dynamic contractile phage tail-like structure. Nature. 2012;483(7388):182-186.

15. Basnak G, Morton VL, Rolfsson O, Stonehouse NJ, Ashcroft AE, Stockley PG. Viral genomic single-stranded RNA directs the pathway toward a T=3 capsid. J Mol Biol. 2010;395(5):924-936.

16. Benson SD, Bamford JK, Bamford DH, Burnett RM. Viral evolution revealed by bacteriophage PRD1 and human adenovirus coat protein structures. Cell. 1999;98(6):825-833.

17. Bernhardt TG, Struck DK, Young R. The lysis protein E of phi X174 is a specific inhibitor of the MraY- catalyzed step in peptidoglycan synthesis. J Biol Chem. 2001;276(9):6093-6097.

18. Bernhardt TG, Wang IN, Struck DK, Young R. A protein antibiotic in the phage Qbeta virion: diversity in lysis targets. Science. 2001;292(5525):2326-2329.

19. Berry J, Summer EJ, Struck DK, Young R. The final step in the phage infection cycle: the Rz and Rz1 lysis proteins link the inner and outer membranes. Mol Microbiol. 2008;70(2):341-351.

20. Bertani G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J Bacteriol. 1951;62(3):293-300.

21. Bhardwaj A, Molineux IJ, Casjens SR, Cingolani G. Atomic structure of bacteriophage Sf6 tail needle knob. J Biol Chem. 2011;286(35):30867-30877.

22. Bowden DW, Modrich P. In vitro maturation of circular bacteriophage P2 DNA. Purification of ter components and characterization of the reaction. J Biol Chem. 1985;260(11):6999-7007.

23. Breitbart M, Felts B, Kelley S, et al. Diversity and population structure of a nearshore marine sediment viral community. Proc R Soc B. 2004;271:565-574.

24. Browning C, Shneider MM, Bowman VD, Schwarzer D, Leiman PG. Phage pierces the host cell membrane with the iron-loaded spike. Structure. 2012;20(2):326-339.

25. Brussow H, Canchaya C, Hardt WD. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. Microbiol Mol Biol Rev. 2004;68(3):560-602.

26. Bukhari AI, Ambrosio L. The invertible segment of bacteriophage Mu DNA determines the adsorption properties of Mu particles. Nature. 1978;271(5645):575-577.

27. Burmeister AR, Turner PE. Trading-off and trading-up in the world of bacteria-phage evolution. Curr Biol. 2020;30(19):R1120-R1124.

28. Burroughs AM, Iyer LM, Aravind L. Comparative genomics and evolutionary trajectories of viral ATP dependent DNA-packaging systems. Genome Dyn. 2007;3:48-65.

29. Burrows LL. Weapons of mass retraction. Mol Microbiol. 2005;57(4):878-888.

30. Cairns J, Stent GS, Watson JD. Phage and the Origins of Molecular Biology. Cold Spring Harbor Laboratory Press; 1992.

31. Campbell A. Episomes. Adv Genet. 1962;11:101-145.

32. Campbell A. Comparative molecular biology of lambdoid phages. Annu Rev Microbiol. 1994;48:193-222.

33. Campbell A. Life in science: Alan Campbell. Bacteriophage. 2012;2(3):137-138.

34. Casas V, Maloy S. Role of bacteriophage-encoded exotoxins in the evolution of bacterial pathogens. Future Microbiol. 2011;6(12):1461-1473.

35. Cascales E, Cambillau C. Structural biology of type VI secretion systems. Philos Trans R Soc Lond B Biol Sci. 2012;367(1592):1102-1111.

36. Casjens S. Prophages and bacterial genomics: what have we learned so far? Mol Microbiol. 2003;49:277-300.

37. Casjens S, Sampson L, Randall S, et al. Molecular genetic analysis of bacteriophage P22 gene 3 product, a protein involved in the initiation of headful DNA packaging. J Mol Biol. 1992;227(4):1086-1099.

38. Casjens SR. Comparative genomics and evolution of the tailed-bacteriophages. Curr Opin Microbiol. 2005;8(4):451-458.

39. Casjens SR. Diversity among the tailed-bacteriophages that infect the Enterobacteriaceae. Res Microbiol. 2008;159(5):340-348.

40. Casjens SR, Hendrix RW. Locations and amounts of major structural proteins in bacteriophage lambda. J Mol Biol. 1974;88(2):535-545.

41. Casjens SR, Molineux IJ. Short noncontractile tail machines: adsorption and DNA delivery by podoviruses. Adv Exp Med Biol. 2012;726:143-179.

42. Casjens SR, Thuman-Commike PA. Evolution of mosaically related tailed bacteriophage genomes seen through the lens of phage P22 virion assembly. Virology. 2011;411(2):393-415.

43. Caspar DL, Klug A. Physical principles in the construction of regular viruses. Cold Spring Harb Symp Quant Biol. 1962;27:1-24.

44. Catalano CE, Cue D, Feiss M. Virus DNA packaging: the strategy used by phage lambda. Mol Microbiol. 1995;16(6):1075-1086.

45. Chan BK, Sistrom M, Wertz JE, Kortright KE, Narayan D, Turner PE. Phage selection restores antibiotic sensitivity in MDR Pseudomonas aeruginosa. Sci Rep. 2016;6:26717.

46. Chan BK, Turner PE, Kim S, Mojibian HR, Elefteriades JA, Narayan D. Phage treatment of an aortic graft infected with Pseudomonas aeruginosa. Evol Med Public Health. 2018;2018(1):60-66.

47. Christie GE, Anders DL, McAlister V, Goodwin TS, Julien B, Calendar R. Identification of upstream sequences essential for activation of a bacteriophage P2 late promoter. J Bacteriol. 2003;185(15):4609-4614.

48. Clark AJ, Inwood W, Cloutier T, Dhillon TS. Nucleotide sequence of coliphage HK620 and the evolution of lambdoid phages. J Mol Biol. 2001;311(4):657-679.

49. Clark DP. Molecular Biology: Understanding the Genetic Revolution. Elsevier Academic Press; 2005.

50. Claverie JM, Abergel C. Mimivirus and its virophage. Annu Rev Genet. 2009;43:49-66.

51. Conway JF, Duda RL, Cheng N, Hendrix RW, Steven AC. Proteolytic and conformational control of virus capsid maturation: the bacteriophage HK97 system. J Mol Biol. 1995;253(1):86-99.

52. Corchero JL, Cedano J. Self-assembling, protein-based intracellular bacterial organelles: emerging vehicles for encapsulating, targeting and delivering therapeutical cargoes. Microb Cell Fact. 2011;10:92.

53. Court DL, Oppenheim AB, Adhya SL. A new look at bacteriophage lambda genetic networks. J Bacteriol. 2007;189(2):298-304.

54. Crowl RM, Boyce RP, Echols H. Repressor cleavage as a prophage induction mechanism: hypersensitivity of a mutant lambda cI protein to

recA-mediated proteolysis. J Mol Biol. 1981;152(4):815-819.

55. d'Herelle F. Sur un microbe invisible antagonist des bacilles dysenterique. C R Acad Sci. 1917;165:373-375.

56. Damali EJ, Nulton J, Turner P, Rohwer F, Salamon P. Heat output by marine microbial and viral communities. J Non-Equilib Thermodyn. 2012;37(3):291-313.

57. Danovaro R, Corinaldesi C, Dell'anno A, et al. Marine viruses and global climate change. FEMS Microbiol Rev. 2011;35(6):993-1034.

58. Davis BM, Kimsey HH, Kane AV, Waldor MK. A satellite phage-encoded antirepressor induces repressor aggregation and cholera toxin gene transfer. EMBO J. 2002;21(16):4240-4249.

59. Davis BM, Waldor MK. Filamentous phages linked to virulence of Vibrio cholerae. Curr Opin Microbiol. 2003;6(1):35-42.

60. Davydova EK, Kazmierczak KM, Rothman-Denes LB. Bacteriophage N4-coded, virion-encapsulated DNA-dependent RNA polymerase. Methods Enzymol. 2003;370:83-94.

61. Dedrick R, Guerrero Bustamante C, Garlena RA, et al. Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*. Nat Med. 2019;25:730-733.

62. Deveau H, Garneau JE, Moineau S. CRISPR/Cas system and its role in phage-bacteria interactions. Annu Rev Microbiol. 2010;64:475-493.

63. Dewey JS, Savva CG, White RL, Vitha S, Holzenburg A, Young R. Micron-scale holes terminate the phage infection cycle. Proc Natl Acad Sci U S A. 2010;107(5):2219-2223.

64. Ding F, Lu C, Zhao W, et al. Structure and assembly of the essential RNA ring component of a viral DNA packaging motor. Proc Natl Acad Sci U S A. 2011;108(18):7357-7362.

65. Dokland T. Molecular piracy: redirection of bacteriophage capsid assembly by mobile genetic elements. Viruses. 2019;11(11):1003.

66. Duda RL, Teschke CM. The amazing HK97 fold: versatile results of modest differences. Curr Opin Virol. 2019;36:9-16.

67. Dudas KC, Kreuzer KN. UvsW protein regulates bacteriophage T4 origin-dependent replication by unwinding R-loops. Mol Cell Biol. 2001;21(8):2706-2715.

68. Ebersbach G, Gerdes K. Plasmid segregation mechanisms. Annu Rev Genet. 2005;39:453-479.

69. Edgar R, Rokney A, Feeney M, et al. Bacteriophage infection is targeted to cellular poles. Mol Microbiol. 2008;68(5):1107-1116.

70. Edgell DR, Gibb EA, Belfort M. Mobile DNA elements in T4 and related phages. Virol J. 2010;7:290.

71. Fane BA, Brentlinger KI, Burch AD, et al. PhiX174 the Microviridae. In: Calendar R, ed. The Bacteriophages. Oxford University Press; 2006:129-143.

72. Fierer N, Breitbart M, Nulton J, et al. Metagenomic and small-subunit rRNA analyses reveal the genetic diversity of bacteria, archaea, fungi, and viruses in soil. Appl Environ Microbiol. 2007;73(21):7059-7066.

73. Fiers W, Contreras R, Duerinck F, et al. Complete nucleotide sequence of bacteriophage MS2 RNA: primary and secondary structure of the replicase gene. Nature. 1976;260(5551):500-507.

74. Fillol-Salom A, Bacarizo J, Alqasmi M, et al. Hijacking the hijackers: *Escherichia coli* pathogenicity islands redirect helper phage packaging for their own benefit. Mol Cell. 2019;75(5):1020-1030 e4.

75. Fokine A, Leiman PG, Shneider MM, et al. Structural and functional similarities between the capsid proteins of bacteriophages T4 and HK97 point to a common ancestry. Proc Natl Acad Sci U S A. 2005;102(20):7163-7168.

76. Gaidelyte A, Vaara M, Bamford DH. Bacteria, phages and septicemia. PLoS One. 2007;2(11):e1145.

77. Geiduschek EP, Kassavetis GA. Transcription of the T4 late genes. Virol J. 2010;7:288.

78. Gottesman ME, Weisberg RA. Little lambda, who made thee? Microbiol Mol Biol Rev. 2004;68(4):796-813.

79. Grahn AM, Butcher SJ, Bamford JK, Bamford DH. PRD1: dissecting the genome, structure, and entry. In: The Bacteriophages. Oxford University Press; 2006:161-170.

80. Grimes S, Ma S, Gao J, Atz R, Jardine PJ. Role of phi29 connector channel loops in late-stage DNA packaging. J Mol Biol. 2011;410(1):50-59.

81. Guerrero-Bustamante CA, Dedrick RM, Garlena RA, Russell DA, Hatfull GF. Toward a phage cocktail for tuberculosis: susceptibility and tuberculocidal action of mycobacteriophages against diverse *Mycobacterium tuberculosis* strains. MBio. 2021;12(3):e00973-21.

82. Guerrero-Ferreira RC, Viollier PH, Ely B, et al. Alternative mechanism for bacteriophage adsorption to the motile bacterium Caulobacter crescentus. Proc Natl Acad Sci U S A. 2011;108(24):9963-9968.

83. Haigh NG, Webster RE. The pI and pXI assembly proteins serve separate and essential roles in filamentous phage assembly. J Mol Biol. 1999;293(5):1017-1027.

84. Hatfull GF, Dedrick RM, Schooley RT. Phage therapy for antibiotic-resistant bacterial infections. Annu Rev Med. 2022;73:197-211.

85. Hatfull GF, Hendrix RW. Bacteriophages and their genomes. Curr Opin Virol. 2011;1(4):298-303.

86. Haq IU, Chaudhry WN, Akhtar MN, Andleeb S, Qadri I. Bacteriophages and their implications on future biotechnology: a review. Virol J. 2012;9:9.

87. Hendrix RW. Symmetry mismatch and DNA packaging in large bacteriophages. Proc Natl Acad Sci U S A. 1978;75(10):4779-4783.

88. Hendrix RW. Bacteriophage assembly. Nature. 1979;277(5693):172-173.

89. Hendrix RW. Bacteriophage genomics. Curr Opin Microbiol. 2003;6(5):506-511.

90. Hendrix RW. Jumbo bacteriophages. Curr Top Microbiol Immunol. 2009;328:229-240.

91. Hendrix RW, Casjens S. Bacteriophage lambda and its genetic neighborhood. In: Calendar R, ed. The Bacteriophages. Oxford Univeristy Press; 2006:409-447.

92. Hendrix RW, Duda RL. Bacteriophage lambda PaPa: not the mother of all lambda phages. Science. 1992;258(5085):1145-1148.

93. Hendrix RW, Johnson JE. Bacteriophage HK97 capsid assembly and maturation. Adv Exp Med Biol. 2012;726:351-363.

94. Hendrix RW, Smith MC, Burns RN, Ford ME, Hatfull GF. Evolutionary relationships among diverse bacteriophages and prophages: all the world's a phage. Proc Natl Acad Sci U S A. 1999;96(5):2192-2197.

95. Hershey AD, Chase M. Independent functions of viral protein and nucleic acid in growth of bacteriophage. J Gen Physiol. 1952;36(1):39-56.

96. Hinton DM. Transcriptional control in the prereplicative phase of T4 development. Virol J. 2010;7:289.

97. Horiuchi K. Initiation mechanisms in replication of filamentous phage DNA. Genes Cells. 1997;2(7):425-432.

98. Huang WM, Ao SZ, Casjens S, et al. A persistent untranslated sequence within bacteriophage T4 DNA topoisomerase gene 60. Science. 1988;239(4843):1005-1012.

99. Huang WM, Joss L, Hsieh T, Casjens S. Protelomerase uses a topoisomerase IB/Y-recombinase type mechanism to generate DNA hairpin ends. J Mol Biol. 2004;337(1):77-92.

100. Hugel T, Michaelis J, Hetherington CL, et al. Experimental test of connector rotation during DNA packaging into bacteriophage phi29 capsids. PLoS Biol. 2007;5(3):e59.

101. Jobichen C, Chakraborty S, Li M, et al. Structural basis for the secretion of EvpC: a key type VI secretion system protein from Edwardsiella tarda. PLoS One. 2010;5(9):e12910.

102. Johnson JE. Virus particle maturation: insights into elegantly programmed nanomachines. Curr Opin Struct Biol. 2010;20(2):210-216.

103. Julien B, Calendar R. Purification and characterization of the bacteriophage P4 delta protein. J Bacteriol. 1995;177(13):3743-3751.

104. Karam JD, Kreuzer KN, Hall DH. The Molecular Biology of Bacteriophage T4. ASM Press; 1994.

105. Karaolis DK, Somara S, Maneval DR Jr, Johnson JA, Kaper JB. A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. Nature. 1999;399(6734):375-379.

106. Katsura I, Hendrix RW. Length determination in bacteriophage lambda tails. Cell. 1984;39(3 Pt 2):691-698.

107. Kihara A, Akiyama Y, Ito K. Host regulation of lysogenic decision in bacteriophage lambda: transmembrane modulation of FtsH (HflB), the cII degrading protease, by HflKC (HflA). Proc Natl Acad Sci U S A. 1997;94(11):5544-5549.

108. Kivela HM, Daugelavicius R, Hankkio RH, Bamford JK, Bamford DH. Penetration of membrane-containing double-stranded-DNA bacteriophage PM2 into Pseudoalteromonas hosts. J Bacteriol. 2004;186(16):5342-5354.

109. Kobiler O, Rokney A, Oppenheim AB. Phage lambda CIII: a protease inhibitor regulating the lysis-lysogeny decision. PLoS One. 2007;2(4):e363.

110. Kongari R, Rajaure M, Cahill J, et al. Phage spanins: diversity, topological dynamics and gene convergence. BMC Bioinformatics. 2018;19(1):326.

111. Koning R, van den Worm S, Plaisier JR, van Duin J, Pieter Abrahams J, Koerten H. Visualization by cryo-electron microscopy of genomic RNA that binds to the protein capsid inside bacteriophage MS2. J Mol Biol. 2003;332(2):415-422.

112. Kreuzer KN, Brister JR. Initiation of bacteriophage T4 DNA replication and replication fork dynamics: a review in the Virology Journal series on bacteriophage T4 and its relatives. Virol J. 2010;7:358.

113. Krupovic M, Bamford DH. Putative prophages related to lytic tailless marine dsDNA phage PM2 are widespread in the genomes of aquatic bacteria. BMC Genomics. 2007;8:236.

114. Krupovic M, Daugelavicius R, Bamford DH. A novel lysis system in PM2, a lipid-containing marine double-stranded DNA bacteriophage. Mol Microbiol. 2007;64(6):1635-1648.

115. Kuhn A, Wickner W. Conserved residues of the leader peptide are essential for cleavage by leader peptidase. J Biol Chem. 1985;260(29):15914-15918.

116. Lander GC, Tang L, Casjens SR, et al. The structure of an infectious P22 virion shows the signal for headful DNA packaging. Science. 2006;312(5781):1791-1795.

117. Landy A. Dynamic, structural, and regulatory aspects of lambda site-specific recombination. Annu Rev Biochem. 1989;58:913-949.

118. Landy A. The lambda integrase site-specific recombination pathway. Microbiol Spectr. 2015;3(2):MDNA3-0051-2014.

119. Lata R, Conway JF, Cheng N, et al. Maturation dynamics of a viral capsid: visualization of transitional intermediate states. Cell. 2000;100(2):253-263.

120. Laurinavicius S, Bamford DH, Somerharju P. Transbilayer distribution of phospholipids in bacteriophage membranes. Biochim Biophys Acta. 2007;1768(10):2568-2577.

121. Lederberg EM, Lederberg J. Genetic studies of lysogenicity in *Escherichia coli*. Genetics. 1953;38(1):51-64.

122. Leiman PG, Arisaka F, van Raaij MJ, et al. Morphogenesis of the T4 tail and tail fibers. Virol J. 2010;7:355.

123. Leiman PG, Basler M, Ramagopal UA, et al. Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. Proc Natl Acad Sci U S A. 2009;106(11):4154-4159.

124. Leung MM, Florizone SM, Taylor TA, Lang AS, Beatty JT. The gene transfer agent of Rhodobacter capsulatus. Adv Exp Med Biol. 2010;675:253-264.

125. Lewis D, Le P, Zurla C, Finzi L, Adhya S. Multilevel autoregulation of lambda repressor protein CI by DNA looping in vitro. Proc Natl Acad Sci U S A. 2011;108(36):14807-14812.

126. Li Q, Shivachandra SB, Zhang Z, Rao VB. Assembly of the small outer capsid protein, Soc, on bacteriophage T4: a novel system for high density display of multiple large anthrax toxins and foreign proteins on phage capsid. J Mol Biol. 2007;370(5):1006-1019.

127. Liao SM, Wu TH, Chiang CH, Susskind MM, McClure WR. Control of gene expression in bacteriophage P22 by a small antisense RNA. I. Characterization in vitro of the Psar promoter and the sar RNA transcript. Genes Dev. 1987;1(2):197-203.

128. Lindsay JA, Ruzin A, Ross HF, Kurepina N, Novick RP. The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. Mol Microbiol. 1998;29(2):527-543.

129. Loeb T. Isolation of a bacteriophage specific for the F plus and Hfr mating types of *Escherichia coli* K-12. Science. 1960;131(3404):932-933.

130. Lorenz SH, Schmid FX. Reprogramming the infection mechanism of a filamentous phage. Mol Microbiol. 2011;80(3):827-834.

131. Malys N, Chang DY, Baumann RG, Xie D, Black LW. A bipartite bacteriophage T4 SOC and HOC randomized peptide display library: detection and analysis of phage T4 terminase (gp17) and late sigma factor (gp55) interaction. J Mol Biol. 2002;319(2):289-304.

132. Maniloff J, Kampo GJ, Dascher CC. Sequence analysis of a unique temperature phage: mycoplasma virus L2. Gene. 1994;141(1):1-8.

133. Mannisto RH, Kivela HM, Paulin L, Bamford DH, Bamford JK. The complete genome sequence of PM2, the first lipid-containing bacterial virus to be isolated. Virology. 1999;262(2):355-363.

134. Marinelli LJ, Piuri M, Swigonova Z, et al. BRED: a simple and powerful tool for constructing mutant and recombinant bacteriophage genomes. PLoS One. 2008;3(12):e3957.

135. Mascarenhas D, Trueheart J, Benedik M, Campbell A. Retroregulation: control of integrase expression by the b2 region of bacteriophages lambda and 434. Virology. 1983;124(1):100-108.

136. Masters M. Generalized transduction. In: Neidhardt FC, ed. Escherichia coli and Salmonella: cellualr and molecular biology. Vol. 2. ASMA Press; 1996:2421-2441.

137. Medhekar B, Miller JF. Diversity-generating retroelements. Curr Opin Microbiol. 2007;10(4):388-395.

138. Medina E, Wieczorek D, Medina EM, Yang Q, Feiss M, Catalano CE. Assembly and maturation of the bacteriophage lambda procapsid: gpC is the viral protease. J Mol Biol. 2010;401(5):813-830.

139. Medina EM, Andrews BT, Nakatani E, Catalano CE. The bacteriophage lambda gpNu3 scaffolding protein is an intrinsically disordered and biologically functional procapsid assembly catalyst. J Mol Biol. 2011;412(4):723-736.

140. Meijer WJ, Horcajadas JA, Salas M. Phi29 family of phages. Microbiol Mol Biol Rev. 2001;65(2):261-287.

141. Mekler V, Minakhin L, Sheppard C, Wigneshweraraj S, Severinov K. Molecular mechanism of transcription inhibition by phage T7 gp2 protein. J Mol Biol. 2011;413(5):1016-1027.

142. Michel-Briand Y, Baysse C. The pyocins of *Pseudomonas aeruginosa*. Biochimie. 2002;84(5-6):499-510.

143. Miller ES, Kutter E, Mosig G, Arisaka F, Kunisawa T, Ruger W. Bacteriophage T4 genome. Microbiol Mol Biol Rev. 2003;67(1):86-156, table of contents.

144. Mindich L. Packaging in dsRNA viruses. Adv Exp Med Biol. 2012;726:601-608.

145. Molineux I. The T7 group. In: Calendar R, ed. The Bacteriophages. Oxford University Press; 2006:277-301.

146. Molineux IJ. No syringes please, ejection of phage T7 DNA from the virion is enzyme driven. Mol Microbiol. 2001;40(1):1-8.

147. Morais MC. The dsDNA packaging motor in bacteriophage o29. Adv Exp Med Biol. 2012;726:511-547.

148. Mueser TC, Hinerman JM, Devos JM, Boyer RA, Williams KJ. Structural analysis of bacteriophage T4 DNA replication: a review in the Virology Journal series on bacteriophage T4 and its relatives. Virol J. 2010;7:359.

149. Mullaney JM, Black LW. Capsid targeting sequence targets foreign proteins into bacteriophage T4 and permits proteolytic processing. J Mol Biol. 1996;261(3):372-385.

150. Mushegian AR. Are there 10(31) virus particles on Earth, or more, or fewer? J Bacteriol. 2020;202(9):e00052-20.

151. Nagler C, Nagler G, Kuhn A. Cysteine residues in the transmembrane regions of M13 procoat protein suggest that oligomeric coat proteins assemble onto phage progeny. J Bacteriol. 2007;189(7):2897-2905.

152. Nilsson A, Haggård-Ljungquist E. The P2-like bacteriophages. In: Calendar R, ed. The Bacteriophages. Oxford University Press; 2005:365-390.

153. Oppenheim AB, Kobiler O, Stavans J, Court DL, Adhya S. Switches in bacteriophage lambda development. Annu Rev Genet. 2005;39:409-429.

154. Paolozzi L, Ghelardini P. The Bacteriophage Mu. In: Calendar R, ed. The Bacteriophages. Oxford University Press; 2006:469-498.

155. Papke RT, Doolittle WF. Phage evolution: new worlds of genomic diversity. Curr Biol. 2003;13(15):R606-R607.

156. Parent KN, Gilcrease EB, Casjens SR, Baker TS. Structural evolution of the P22-like phages: comparison of Sf6 and P22 procapsid and virion architectures. Virology. 2012;427(2):177-188.

157. Peachman KK, Li Q, Matyas GR, et al. Anthrax vaccine antigen-adjuvant formulations completely protect New Zealand white rabbits against challenge with Bacillus anthracis Ames strain spores. Clin Vaccine Immunol. 2012;19(1):11-16.

158. Pedulla ML, Ford ME, Houtz JM, et al. Origins of highly mosaic mycobacteriophage genomes. Cell. 2003;113(2):171-182.

159. Pedulla ML, Ford ME, Karthikeyan T, et al. Corrected sequence of the bacteriophage P22 genome. J Bacteriol. 2003;185(4):1475-1477.

160. Pell LG, Kanelis V, Donaldson LW, Howell PL, Davidson AR. The phage lambda major tail protein structure reveals a common evolution for long-tailed phages and the type VI bacterial secretion system. Proc Natl Acad Sci U S A. 2009;106(11):4160-4165.

161. Petrov VM, Ratnayaka S, Nolan JM, Miller ES, Karam JD. Genomes of the T4-related bacteriophages as windows on microbial genome evolution. Virol J. 2010;7:292.

162. Ploss M, Kuhn A. Kinetics of filamentous phage assembly. Phys Biol. 2010;7(4):045002.

163. Ploss M, Kuhn A. Membrane insertion and assembly of epitope-tagged gp9 at the tip of the M13 phage. BMC Microbiol. 2011;11:211.

164. Polissi A, Goffin L, Georgopoulos C. The *Escherichia coli* heat shock response and bacteriophage lambda development. FEMS Microbiol Rev. 1995;17(1-2):159-169.

165. Poranen MM, Bamford DH. Assembly of large icosahedral double-stranded RNA viruses. Adv Exp Med Biol. 2012;726:379-402.

166. Poranen MM, Tuma R, Bamford DH. Assembly of double-stranded RNA bacteriophages. Adv Virus Res. 2005;64:15-43.

167. Rao VB, Black LW. Structure and assembly of bacteriophage T4 head. Virol J. 2010;7:356.

168. Rao VB, Feiss M. The bacteriophage DNA packaging motor. Annu Rev Genet. 2008;42:647-681.

169. Rapoza MP, Webster RE. The products of gene I and the overlapping in-frame gene XI are required for filamentous phage assembly. J Mol Biol. 1995;248(3):627-638.

170. Ravin NV. Mechanisms of replication and telomere resolution of the linear plasmid prophage N15. FEMS Microbiol Lett. 2003;221(1):1-6.

171. Ravin NV. N15: the linear phage-plasmid. Plasmid. 2011;65(2):102-109.

172. Ren ZJ, Lewis GK, Wingfield PT, Locke EG, Steven AC, Black LW. Phage display of intact domains at high copy number: a system based on SOC, the small outer capsid protein of bacteriophage T4. Protein Sci. 1996;5(9):1833-1843.

173. Reyes A, Semenkovich NP, Whiteson K, Rohwer F, Gordon JI. Going viral: next-generation sequencing applied to phage populations in the human gut. Nat Rev Microbiol. 2012;10(9):607-617.

174. Richet E, Abcarian P, Nash HA. Synapsis of attachment sites during lambda integrative recombination involves capture of a naked DNA by a protein-DNA complex. Cell. 1988;52(1):9-17.

175. Roberts JW. Phage lambda and the regulation of transcription termination. Cell. 1988;52(1):5-6.

176. Roberts JW, Yarnell W, Bartlett E, et al. Antitermination by bacteriophage lambda Q protein. Cold Spring Harb Symp Quant Biol. 1998;63:319-325.

177. Rodriguez-Brito B, Li L, Wegley L, et al. Viral and microbial community dynamics in four aquatic environments. ISME J.

2010;4(6):739-751.

178. Roessner CA, Ihler GM. Proteinase sensitivity of bacteriophage lambda tail proteins gpJ and pH in complexes with the lambda receptor. J Bacteriol. 1984;157(1):165-170.

179. Roessner CA, Struck DK, Ihler GM. Injection of DNA into liposomes by bacteriophage lambda. J Biol Chem. 1983;258(1):643-648.

180. Russel M, Linderoth NA, Sali A. Filamentous phage assembly: variation on a protein export theme. Gene. 1997;192(1):23-32.

181. Russel M, Model P. Genetic analysis of the filamentous bacteriophage packaging signal and of the proteins that interact with it. J Virol. 1989;63(8):3284-3295.

182. Salas M. Phage phi21 and its relatives. In: Calendar R, ed. The Bacteriophages. Oxford Press; 2006:315-330.

183. Samuelson JC, Chen M, Jiang F, et al. YidC mediates membrane protein insertion in bacteria. Nature. 2000;406(6796):637-641.

184. Sandmeier H. Acquisition and rearrangement of sequence motifs in the evolution of bacteriophage tail fibres. Mol Microbiol. 1994;12(3):343-350.

185. Sanger F, Coulson AR, Hong GF, Hill DF, Petersen GB. Nucleotide sequence of bacteriophage lambda DNA. J Mol Biol. 1982;162(4):729-773.

186. Sathaliyawala T, Rao M, Maclean DM, Birx DL, Alving CR, Rao VB. Assembly of human immunodeficiency virus (HIV) antigens on bacteriophage T4: a novel in vitro approach to construct multicomponent HIV vaccines. J Virol. 2006;80(15):7688-7698.

187. Sauer B. Inducible gene targeting in mice using the Cre/lox system. Methods. 1998;14(4):381-392.

188. Schooley RT, Biswas B, Gill JJ, et al. Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant Acinetobacter baumannii infection. Antimicrob Agents Chemother. 2017;61(10):e00954-17.

189. Schwarzer D, Buettner FF, Browning C, et al. A multivalent adsorption apparatus explains the broad host range of phage phi92: a comprehensive genomic and structural analysis. J Virol. 2012;86(19):10384-10398.

190. Selivanovitch E, Douglas T. Virus capsid assembly across different length scales inspire the development of virus-based biomaterials. Curr Opin Virol. 2019;36:38-46.

191. Sharma J, Uchida M, Miettinen HM, Douglas T. Modular interior loading and exterior decoration of a virus-like particle. Nanoscale. 2017;9(29):10420-10430.

192. Smith DE, Tans SJ, Smith SB, Grimes S, Anderson DL, Bustamante C. The bacteriophage straight phi29 portal motor can package DNA against a large internal force. Nature. 2001;413(6857):748-752.

193. Smith GP. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science. 1985;228(4705):1315-1317.

194. Stewart CR, Casjens SR, Cresawn SG, et al. The genome of *Bacillus subtilis* bacteriophage SPO1. J Mol Biol. 2009;388(1):48-70.

195. Stromsten NJ, Bamford DH, Bamford JK. The unique vertex of bacterial virus PRD1 is connected to the viral internal membrane. J Virol. 2003;77(11):6314-6321.

196. Sumida-Yasumoto C, Ikeda JE, Benz E, et al. Replication of phiX174 DNA: in vitro synthesis of phiX RFI DNA and circular, single-stranded DNA. Cold Spring Harb Symp Quant Biol. 1979;43 Pt 1:311-329.

197. Summers WC. Felix d'Herelle and the Origins of Molecular Biology. Yale University Press; 1999.

198. Sun S, Kondabagil K, Draper B, et al. The structure of the phage T4 DNA packaging motor suggests a mechanism dependent on electrostatic forces. Cell. 2008;135(7):1251-1262.

199. Susskind MM, Botstein D. Molecular genetics of bacteriophage P22. Microbiol Rev. 1978;42(2):385-413.

200. Suttle CA. Marine viruses—major players in the global ecosystem. Nat Rev Microbiol. 2007;5(10):801-812.

201. Tao P, Li Q, Shivachandra SB, Rao VB. Bacteriophage T4 as a nanoparticle platform to display and deliver pathogen antigens: construction of an effective anthrax vaccine. Methods Mol Biol. 2017;1581:255-267.

202. Tay LL, Huang PJ, Tanha J, et al. Silica encapsulated SERS nanoprobe conjugated to the bacteriophage tailspike protein for targeted detection of Salmonella. Chem Commun (Camb). 2012;48(7):1024-1026.

203. Taylor K, Wegrzyn G. Replication of coliphage lambda DNA. FEMS Microbiol Rev. 1995;17(1-2):109-119.

204. Tucker KP, Parsons R, Symonds EM, Breitbart M. Diversity and distribution of single-stranded DNA phages in the North Atlantic Ocean. ISME J. 2011;5(5):822-830.

205. Twort F. An investigation on the nature of ultramicroscopic viruses. Lancet. 1915;189:1241-1243.

206. Tyler JS, Livny J, Friedman DI. Lambdoid phages and shiga toxin. In: Waldor MK, Friedman DI, Adhya S, eds. Phages: Role in Pathogenesis and Biotechnology. ASM Press; 2005.

207. Uzan M, Miller ES. Post-transcriptional control by bacteriophage T4: mRNA decay and inhibition of translation initiation. Virol J. 2010;7(1):360.

208. van Dijk AA, Makeyev EV, Bamford DH. Initiation of viral RNA-dependent RNA polymerization. J Gen Virol. 2004;85(Pt 5):1077-1093.

209. van Duin J, Tsareva N. Single-stranded RNA Phages. In: Calendar R, ed. The Bacteriophages. Oxford University Press; 2006:175-196.

210. Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science. 1996;272(5270):1910-1914.

211. Wang IN, Smith DL, Young R. Holins: the protein clocks of bacteriophage infections. Annu Rev Microbiol. 2000;54:799-825.

212. Webster RE. Biology of the filamentous bacteriophage. In: Kay BK, Winter J, McCaffrey J, eds. Phage Display of Peptides and Proteins: A Laboratory Manual. Academic Press; 1996.

213. Weigel C, Seitz H. Bacteriophage replication modules. FEMS Microbiol Rev. 2006;30(3):321-381.

214. Werts C, Michel V, Hofnung M, Charbit A. Adsorption of bacteriophage lambda on the LamB protein of *Escherichia coli* K-12: point mutations in gene J of lambda responsible for extended host range. J Bacteriol. 1994;176(4):941-947.

215. Wetzel KS, Guerrero-Bustamante CA, Dedrick RM, et al. CRISPY-BRED and CRISPY-BRIP: efficient bacteriophage engineering. Sci Rep. 2021;11(1):6796.

216. Wichman HA, Scott LA, Yarber CD, Bull JJ. Experimental evolution recapitulates natural evolution. Philos Trans R Soc Lond B Biol Sci. 2000;355(1403):1677-1684.

217. Wikoff WR, Liljas L, Duda RL, Tsuruta H, Hendrix RW, Johnson JE. Topologically linked protein rings in the bacteriophage HK97 capsid. Science. 2000;289(5487):2129-2133.

218. Wilcox RM, Fuhrman JA. Bacterial viruses in coastal seawater: lytic rather than lysogenic production. Mar Ecol Prog Ser. 1994;114:35-45.

219. Wilhelm SW, Brigden SM, Suttle CA. A dilution technique for the direct measurement of viral production: a comparison in stratified and tidally mixed coastal waters. Microb Ecol. 2002;43(1):168-173.

220. Willner D, Furlan M, Schmieder R, et al. Metagenomic detection of phage-encoded platelet-binding factors in the human oral cavity. Proc Natl Acad Sci U S A. 2011;108(Suppl 1):4547-4553.

221. Woodhead GS. Koch's recent researches on tuberculin. Nature. 1897;55:567.

222. Wu R, Kaiser AD. Structure and base sequence in the cohesive ends of bacteriophage lambda DNA. J Mol Biol. 1968;35(3):523-537.

223. Xu J, Hendrix RW, Duda RL. Conserved translational frameshift in dsDNA bacteriophage tail assembly genes. Mol Cell. 2004;16(1):11-21.

224. Yarmolinsky MB, Sternberg N. Bacteriophage P1. In: Calendar R, ed. The Bacteriophages. Vol. 2. Plenum Press; 1988:291-438.

225. Young R. Bacteriophage lysis: mechanism and regulation. Microbiol Rev. 1992;56(3):430-481.

226. Yu A, Haggard-Ljungquist E. The Cox protein is a modulator of directionality in bacteriophage P2 site-specific recombination. J Bacteriol. 1993;175(24):7848-7855.

227. Zaldastanishvili E, Leshkasheli L, Dadiani M, et al. Phage therapy experience at the Eliava Phage Therapy Center: three cases of bacterial persistence. Viruses. 2021;13(10):1901.

228. Zheng W, Wang F, Taylor NMI, Guerrero-Ferreira RC, Leiman PG, Egelman EH. Refined Cryo-EM structure of the T4 tail tube: exploring the lowest dose limit. Structure. 2017;25(9):1436-1441 e2.

229. Zhu J, Ananthaswamy N, Jain S, et al. A universal bacteriophage T4 nanoparticle platform to design multiplex SARS-CoV-2 vaccine candidates by CRISPR engineering. Sci Adv. 2021;7(37):eabh1547.

230. Zinder ND, Lederberg J. Genetic exchange in Salmonella. J Bacteriol. 1952;64(5):679-699.

CHAPTER 22

Prions

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Acknowledgments

Prions Are Alternative, Transmissible States of Proteins

Prions are protein-based infectious or transmissible agents that, unlike viruses and other pathogens, lack a specific nucleic acid genome.[312] Prominent examples of prion diseases in mammals include Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, and chronic wasting disease (CWD) in cervids such as deer and elk. Usually, prions are nonnative forms of host proteins that replicate themselves by converting their normal counterpart to the prion state. As has been described in fungi, prions also include epigenetic elements of inheritance comprising altered forms of host proteins that, when transferred to a new host, can replicate and cause a heritable phenotypic change in the recipient (see Chapter 20).[408] Most prions cause disease in the host, but some have physiological roles.[409] Although prions do not carry any nucleic acid code for themselves, a susceptible host must make the normal precursor protein from which the infectious prions are made. Many proteins can refold and/or assemble into ordered aggregates that can seed further growth under certain natural or experimental conditions, whether *in vivo* or *in vitro*.[94] Indeed, many normal macromolecular assemblies within organisms grow by such mechanisms. However, most such protein assemblies are not, strictly speaking, prions because the prion concept implies that the altered state of the protein can propagate between individuals, or at least from cell-to-cell within a multicellular host. In this chapter, we review many aspects of mammalian prion structure and biology but, given space limitations, we have only been able to include representative primary references or reviews of the literature.

Evolution of the Prion Concept

Perhaps the earliest descriptions of a prion disease were in the 1700s when scrapie was observed as a peculiar fatal affliction of sheep.[54] An obvious manifestation of scrapie was a desire to scrape against abrasive objects, presumably due to itching in the skin. Later studies early in the 20th century indicated that scrapie is transmissible and can remain contagious in vacant pastures for years. Scrapie infectivity was found to be unusually resistant to treatments that destroy other types of pathogens, leading to proposals in the 1960s that it may be an abnormal self-replicating form of a protein or membrane that lacks its own nucleic acid genome.[7,161,305] In 1957, Vincent Zigas and D. Carleton Gajdusek travelled to Papua New Guinea to study a rapidly fatal neurodegenerative disease affecting the Foré people. This disease was called "kuru," which means "to shiver," as the symptoms included startle myoclonus. It was primarily affecting women and children, and the cause was unknown. A neuropathology exhibit on brains from kuru patients was presented in London in 1959, where William Hadlow, a veterinarian pathologist, attended. Hadlow had experience with the transmissible spongiform encephalopathy "scrapie," which affects sheep, and he noted the similarity between the spongiform changes seen in kuru brains and those seen in scrapie. He proposed that kuru might also be transmissible.[164] This was subsequently proven for both kuru[141] and CJD.[152] It was then determined that the transmission of kuru was through funeral cannibalistic practices, with higher titer tissues (brain) being preferentially given to women and children, hence the higher incidence in these groups.[229] These observations earned Gajdusek a Nobel Prize in 1976. Complicating epidemiological studies of kuru and other prion diseases are the long incubation periods between the initial infection and the appearance of clinical signs, which, in humans, can exceed four decades. Such prolonged incubation times, together with the small minimum size of the infectious particles, led many in the field at the time to refer initially to these agents as "slow viruses."

In 1981, Patricia Merz and colleagues identified abnormal scrapie-associated fibrils (SAF) in subfractions of brain from scrapie-infected animals.[273] Soon thereafter, Stanley Prusiner and colleagues identified a protease-resistant protein ("PRP") that turned out to be the main component of both SAF and scrapie infectivity,[47,315] which Prusiner dubbed *prions*.[47,311,315] PRP then became PrP, an abbreviation for prion protein. The Prusiner, Charles Weissmann, and Bruce Chesebro laboratories cloned the PrP gene (*Prnp*) from hamsters and mice and showed that it was present in both infected and uninfected rodents.[88,235,289] PrP was then shown to undergo a posttranslational conversion in infected hosts from a detergent-soluble, mainly monomeric, protease-sensitive, and α-helical form (named PrP^{C} for "cellular PrP," details to follow) into a usually aggregated, insoluble, and partially protease-resistant state that is high in β-sheet structure (named PrP^{Sc} for "PrP-scrapie") (Fig. 22.1).[49,79,82,300] Additional studies identified many mammalian homologs of PrP that are the main components of abnormal aggregates associated with other transmissible scrapielike neurodegenerative disorders of humans and other animals.[267] These diseases are now known as prion diseases or, with reference to the frequently observed spongelike neuropathological lesions, transmissible spongiform encephalopathies (TSEs).

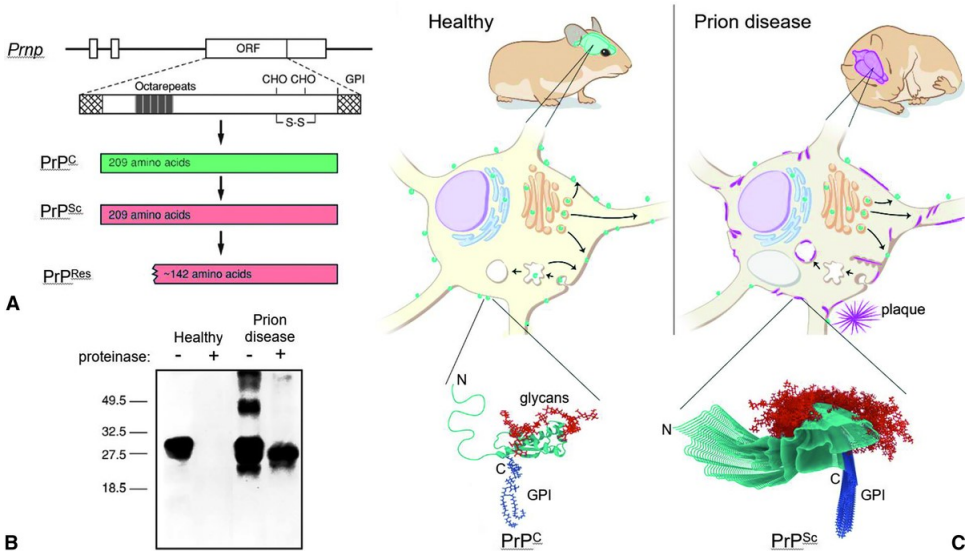

FIGURE 22.1 **Prion protein isoforms**. **A**. Diagram of the Syrian hamster PrP (prion protein) gene (*Prnp*) that encodes a protein of 254 amino acid residues. After biosynthetic processing of the N- and C-termini, both PrP^{C} (PrP-cellular) and PrP^{Sc} (PrP-scrapie) consist of 209 residues. Among the basic features of mammalian PrP molecules are a series of octapeptide repeats (octarepeats), a disulfide bond (S-S), two N-linked glycosylation sites (CHO), and a C-terminal glycophosphatidylinositol (GPI) anchor. After limited proteolysis of PrP^{Sc}, a core of ~142 residues (PrP^{Res}) remains. **B**. Immunoblot of brain homogenates from healthy and prion-diseased hamsters with and without prior proteinase K treatment. PrP^{C} was completely degraded as shown in the healthy homogenate, whereas only ~67 residues were removed from the N-terminus of PrP^{Sc}. Molecular weight markers (kD) are located on the *left*. **C**. In healthy mammals, PrP^{C} (*aqua*) is usually a membrane-bound monomer expressed in many cell types but particularly abundant in neurons. It has a flexible N-terminal domain (drawn shorter than actual scale relative to rest of molecule) and a folded C-terminal domain with three helices, up to two glycans (*red*), and a GPI membrane anchor (*blue*) on the end. In diseased mammals, monomeric PrP^{C} is converted to PrP^{Sc} (*magenta* in the cell cartoon), which is usually found as membrane-bound multimers (see later sections) but can sometimes escape the membrane environment to form extracellular plaques. (**A** and **B** adapted from Figure 76.1A and B in Prusiner SB. Prions. In: Knipe DM, Howley P, eds. *Fields Virology*. Vol 2. 6th ed. Wolters Kluwer Health/Lippincott Williams & Wilkins; 2013; **C** adapted from the graphical abstract and Figure 4B of Kraus A, Hoyt F, Schwartz CL, et al. High-resolution structure and strain comparison of infectious mammalian prions. *Mol Cell*. 2021;81(21):4540-4551. e6. Copyright © 2021 Elsevier, with permission.)

Prion Replication and Strains

The evidence for PrP being fundamental to prion disease continued to build, with Weissmann and others showing that PrP is an essential susceptibility factor for prion diseases.[60] Prusiner won a Nobel Prize in 1997 for his contributions. In addition, human familial prion diseases were linked to expression of various mutant PrPs (see below). Yet the fact that prion diseases could present with distinct phenotypes, neuropathologies and incubation periods led to the recognition of distinct prion strains.[58,197] Furthermore, the consistent propagation of divergent prion strains in hosts with the same PrP sequence raised the question of what "encodes" prion strain characteristics when the gene involved is invariant.

Early in the 1990s, Richard Bessen and Richard Marsh reported evidence that abnormal PrP aggregates that accumulated in hamsters infected with different prion strains had different conformations, providing a potential basis for prion strain variation.[44] Concurrently, the Byron Caughey and Peter Lansbury laboratories found collaboratively that disease-associated aggregated forms of PrP could themselves induce the conversion of normal PrP molecules to abnormal forms *in vitro*.[74,204] These findings provided direct biochemical evidence of the self-replicating activity of the prions. Such prion-induced conversion reactions also exhibited profound biochemical specificities that suggested molecular mechanisms for known prion strain characteristics and transmission barriers between different host species.[43,205,324] However, the yields of the initial cell-free PrP conversion reactions were substoichiometric, and the subsequent development of continuous cell-free prion amplification or *de novo* prion formation reactions, primarily by the Claudio Soto, Surachai Supattapone, and Prusiner laboratories in the 2000s, was crucial to prove unequivocally that prions were composed of refolded PrP aggregates without any requirement for prion-specific, coding nucleic acids.[71,98,118,223,334] There are now many lines of evidence that support the hypothesis that faithful propagation of distinct PrP^{Sc} conformers underpins the existence of prion strains within a given type of host.[33,122,212,352] There is also evidence that nonprotein cofactors, for example, nonspecific nucleic acids, sulfated glycosaminoglycans, or phospholipids, can influence PrP conformational conversions[80,117,140,169,371,402,417,419] and modulate host susceptibilities or disease phenotypes. Notably, Suehiro Sakaguchi and colleagues have reported that infection of murine neuroblastoma cells with a neurotropic H5N1 avian influenza A virus can instigate the formation of PrP^{Sc} that can then propagate continuously on its own and be transmissible to mice.[169]

Extension of the Prion Paradigm to Fungi

Although the name "prion" was initially given to the agents of the TSEs as described above, the first clear evidence that transmissible protein states exist in biology came from Reed Wickner's demonstration that unexplained epigenetic elements of yeast were prions.[407] These prions were not composed of a yeast homolog of PrP but of entirely distinct proteins (see Chapter 20).

Extension to Other Mammalian Protein Misfolding Diseases

Analogous to PrP-based prion disorders, many diseases of mammals involve the formation and prionlike spreading of specific protein aggregates within the host. Prominent examples in humans include Alzheimer disease (Aβ peptide and tau protein); Parkinson disease (α-synuclein), chronic traumatic encephalopathy (tau), type 2 diabetes (islet amyloid polypeptide), and amyotrophic lateral sclerosis (TAR DNA-binding protein-43, superoxide dismutase 1, and fused in sarcoma).[94,211,262,361,398] Studies of several of these proteinopathies have shown that extracts of human patients can induce, or at least accelerate, related pathogenesis in experimental animals. Such findings have fueled concerns that, like TSEs, these diseases might be transmissible and therefore are examples of prion, or prionlike, diseases. The extent to which transmitted etiologies of these various protein aggregation diseases might be clinically significant is currently under debate[18] (see Perspectives section). However, at present, it appears that the vast majority of cases of such human proteinopathies are of sporadic or genetic, rather than acquired, origin.[181]

OVERVIEW OF PrP PRIONS

This chapter will focus primarily on PrP-based TSE prions where a host's normal PrP is converted to aberrant conformers that are typically aggregated.[73,211] Terminology in the prion field is evolving and variably applied (Table 22.1), but here, we will use the term prion, by itself, to refer to the infectious agents of prion diseases; PrP^{Sc}, generically, for forms of PrP that are known to be infectious; and PrP^{Res} to designate protease-resistant forms of PrP^{Sc}. Otherwise, we will use the term PrP^{d} to refer to any disease-associated form of PrP[189] and PrP^{C} for the normal, cellular, nonprion, form.[61]

TABLE 22.1 Prion-related glossary

Term	Description
Prion	A proteinaceous infectious particle that lacks a self-coding nucleic acid and can self-propagate
PrP	Prion protein (any isoform of *PRNP* or *Prnp*-encoded polypeptide)
PrP^{Sc}	Abnormal, pathogenic isoform of PrP that causes illness (PrP-scrapie); the key protein component of transmissible agent of the PrP-based prion diseases or transmissible spongiform encephalopathies
PrP^{C}	Normal cellular isoform of PrP
PrP^{d}	Disease-associated form of PrP, whether transmissible, pathogenic, or not
PrP^{Res}	Partially protease-resistant form of PrP^{Sc}
PrP^{Sen}	Protease-sensitive form of PrP
PRNP	Human PrP gene located on chromosome 20
Prnp	Mouse PrP gene located on syntenic chromosome 2
PIRIBS	Parallel in-register intermolecular β-sheet
Amyloid fibril	Filamentous polypeptide assembly with high cross-β-sheet secondary structure that typically stains with amyloid-sensitive dyes such as Congo red, thioflavin T, and thioflavin S
Amyloid plaque	Localized deposit of many amyloid fibrils in tissue
Prion strain	Prion isolate causing consistent combination of clinical signs, pathological lesions, and PrP^{Res} subtype after inoculation into a given type of host. Incubation periods can be highly predictable and strain-dependent with defined doses and routes of inoculation
GPI	Glycophosphatidylinositol

PrP Prion Structures

A variety of aberrant forms of PrP have been observed *in vivo* and *in vitro*, and a major challenge is to understand which forms are relevant to

disease. Histological staining of infected brain tissue with PrP antibodies has revealed that PrP^d deposits can range from small, diffuse deposits to large extracellular amyloid plaques (Fig. 22.2A and B). The relative amounts and distributions of PrP^d deposits can vary systematically with prion strain and host type.[33,87,90,149,189] Immunogold labeling with transmission electron microscopy has shown that PrP^d can often accumulate in forms that are not visibly fibrillar,[73,189] with the close association of PrP^d with cellular membranes obscuring visualization of PrP^d morphology (Fig. 22.2C-G).

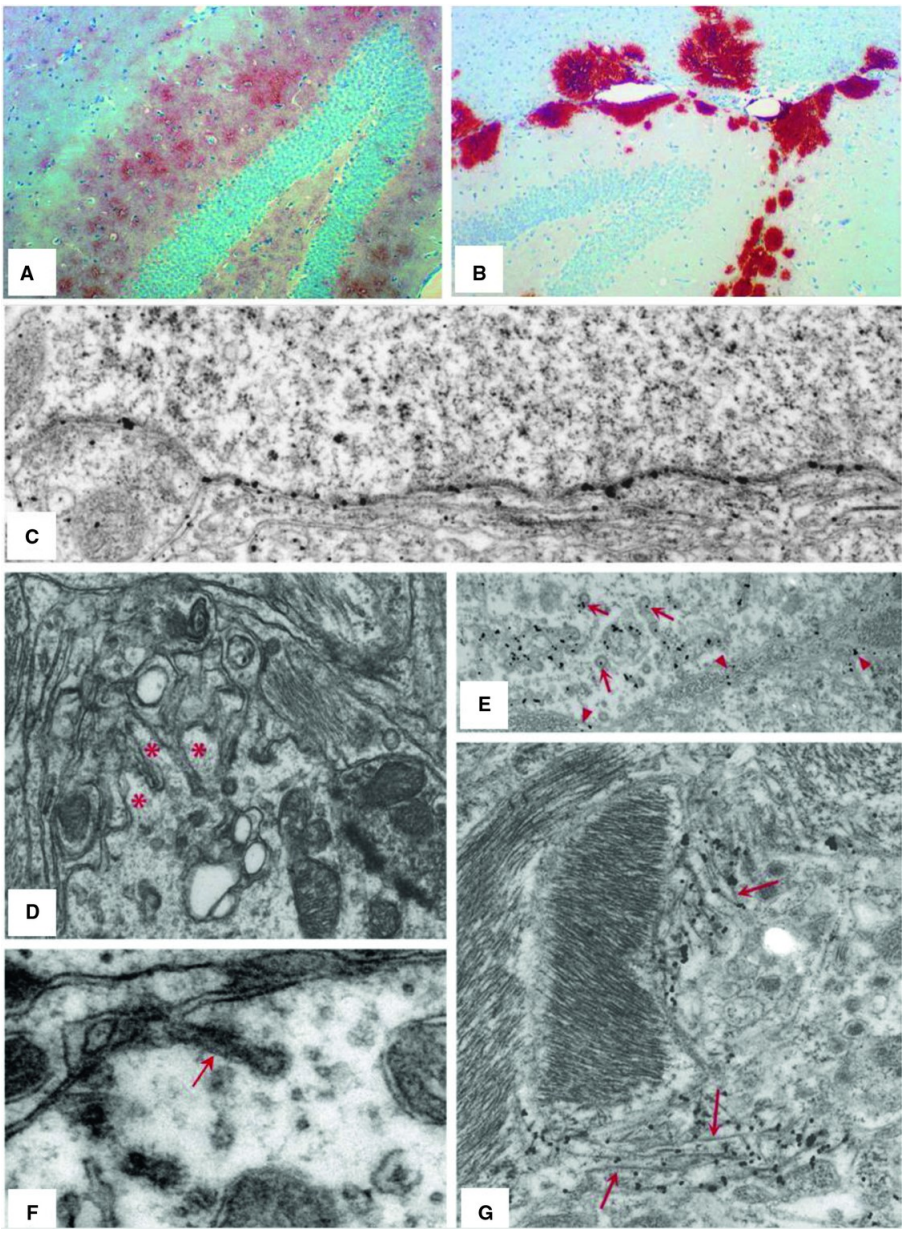

FIGURE 22.2 **Abnormal PrP^d deposition in prion-infected brain tissue**. **A**. Immunohistochemical staining for PrP^d (*red*) in scrapie-infected wild-type mice (diffuse staining) and **(B)** mice expressing only GPI-anchorless PrP (amyloid plaques). **C**. Immunogold labeling of PrP^d accumulation *without* membrane distortions on the surface on neurons and astrocytes in scrapie-infected sheep brain tissue. **D**. Dendritic membrane distortions (microfolding labeled with *asterisks*), not immunolabeled. **E**. Immunolabeled PrP^d associated with excess coated pits (*arrows*) and transfer to adjacent processes (*arrowheads*). **F**. Coated membrane invaginations, including one with a twisted neck (*arrow*), not immunolabeled. **G**. Astrocytic plasma membrane with PrP^d labeling adjacent to abundant glial filaments. *Arrows* designate linear segments of plasmalemma suggesting increased membrane rigidity and nascent fibril formation. (Panels **A and B** adapted from Figure 1A,B by permission from Nature: Caughey B, Baron GS. Prions and their partners in crime. *Nature*. 2006;443(7113):803-810. Copyright © 2006 Springer Nature; and Figure 5 of Caughey B, Baron GS, Chesebro B, et al. Getting a grip on prions: oligomers, amyloids, anchors and pathological membrane interactions. *Annu Rev Biochem*. 2009;78:177-204. Panels **C-G** adapted with permission of Annual Reviews, Inc. from Figure 8A-E of Caughey B, Baron GS, Chesebro B, et al. Getting a grip on prions: oligomers, amyloids, anchors and pathological membrane interactions. *Annu Rev Biochem*. 2009;78:177-204; permission conveyed through Copyright Clearance Center, Inc. and adapted from Figure 2A,B of Jeffrey M, McGovern G, Goodsir CM, et al. Strain-associated variations in abnormal PrP trafficking of sheep scrapie. *Brain Pathol*. 2009;19(1):1-11. Reprinted by permission of John Wiley & Sons, Inc.)

Upon extraction from tissues with detergents, PrP^d most often is found in the form of amyloid fibrils.[273] However two-dimensional, sheetlike crystalline arrays and other nonfibrillar or subfibrillar oligomeric ultrastructures have also been observed (Fig. 22.3).[106,356,393,413] Both

fibrillar and smaller oligomeric forms have been linked to prion infectivity,[106,337,356,386,393] but the relative pathological roles of various prion ultrastructures remain to be determined. Here, we will focus primarily on the structures of fibrillar forms of PrP, including PrP^{Sc}/PrP^{Res}, because those are the only forms for which high-resolution structural data are available.

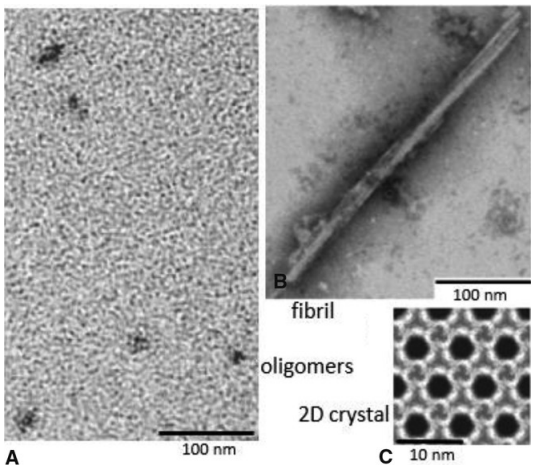

FIGURE 22.3 **Extremes of PrP^{d} ultrastructures**. **A**. Negative stained EM images of particles in a highly infectious sample at the smallest end of a hamster PrP^{Sc} size fractionation.[355,356] **B**. Murine PrP^{Sc} fibril. **C**. Electron crystallography image from two-dimensional (2D) crystals of murine RML scrapie PrP^{Res} preparation.[412] (Panel **A** adapted from Figure 3 (fraction 10) of Silveira JR, Raymond GJ, Hughson AG, et al. The most infectious prion protein particles. *Nature*. 2005;437:257-261. Panel **C** adapted from Figure 3E of Wille H, Govaerts C, Borovinskiy A, et al. Electron crystallography of the scrapie prion protein complexed with heavy metals. *Arch Biochem Biophys*. 2007;467(2):239-248. Copyright © 2007 Elsevier, with permission; and Figure 76.7A Prusiner SB. Prions. In: Knipe DM, Howley P, eds. *Fields Virology*. Vol 2. 6th ed. Wolters Kluwer Health/Lippincott Williams & Wilkins; 2013.)

The properties of prions have prevented determination of their detailed structures by solid state nuclear magnetic resonance (ssNMR) spectroscopy or conventional x-ray or electron diffraction techniques. Nonetheless, fiber diffraction data from infectious brain-derived prion fibrils have revealed regular ~4.9 Å spacings of polypeptide strands perpendicular to the fibril axis; this spacing is characteristic of amyloid fibrils formed by stacked β-strands.[35] Electron paramagnetic resonance (EPR) and ssNMR studies of the protease-resistant cores of synthetic recombinant PrP fibrils have also demonstrated that PrP molecules, or fragments thereof, can form fibrils in which monomers are stacked parallel and in-register (ie, residues of one monomer are aligned with the corresponding residues of the adjacent monomers)[97,162,380,385] (Fig. 22.4A). This alignment, together with the ~4.9 Å spacing, showed that these synthetic PrP fibrils have parallel in-register intermolecular β-sheet/stack (PIRIBS) architectures. These and many other empirically derived, but relatively low-resolution, conformational constraints on PrP fibrils underpinned initial proposals of PIRIBS-based molecular models of prion fibrils.[162]

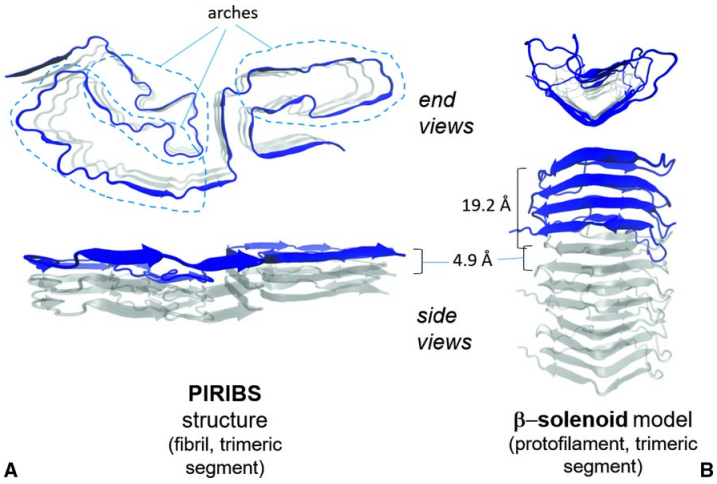

FIGURE 22.4 **Parallel in-register intermolecular β-sheet/stack (PIRIBS) vs 4-rung β-solenoid (4RβS) architectures for prion fibrils**. **A**. Short segment (trimer) of PIRIBS-based architecture as shown by high-resolution cryo-EM analysis of 263K prions.[212] **B**. Segment (trimer) of 4RβS protofilament model more speculatively based on low-resolution cryo-EM data for GPI-anchorless RML prion.[362] *Top* models show cross-sectional views. *Bottom* models show lateral views. A single monomeric unit within each segment is highlighted in *blue*. These models were each drawn using publicly available PDB coordinates as specified in Refs.[212,362] (Figure assembled from modeling by Efrosini Artikis. Adapted from Artikis E, Kraus A, Caughey B. Structural biology of ex vivo mammalian prions. *J Biol Chem*. 2022;298(8):102181. Copyright ©2022 Elsevier, with permission.)

An alternative model arose from brain-derived prion fiber diffraction patterns and low-resolution cryo-electron microscopy (cryo-EM) data that have been interpreted by some groups to indicate a 4-rung β-solenoid (4RβS)-based architecture.[362,395] Among the salient clues was evidence of repeated spacing along the fibril axis of 19.2 Å in addition to the ~4.9 Å spacing between strands within β-sheets. In the proposed 4RβS architectures, each PrP molecule provides four adjacent rungs along the protofilament axis (Fig. 22.4B).

Recent cryo-EM studies have further characterized the types of structural motifs that can be formed by PrP fragments within synthetic PIRIBS fibrils. These fibrils contained two closely packed symmetrical protofilaments, each with a β-arch (a hairpinlike loop of residues with PIRIBS structures on its flanks) at its core.[142,156,401] Analogous hairpinlike motifs in various PrP fibrils may not meet all formal β-arch criteria, so we have taken to simply calling them arches. While these synthetic fibrils are likely to be noninfectious, or at least orders of magnitude less infectious than many natural prions,[75,163,213,226] studies of their distinct modes of assembly within the PIRIBS framework set the stage for the most recent structural analyses of infectious fibrils.

Near-Atomic Structures of Infectious Tissue-Derived Prion Fibrils

Three near-atomic cryo-EM–based structures for fully infectious tissue-derived prion fibrils are available as of this writing, each of which have PIRIBS-based architectures. The first to be solved was PrP^{Res} fibrils of the hamster-adapted scrapie strain 263K[212] (Fig. 22.5), which contain not only the full complement of glycans and GPI-anchors of most natural prions but also ~10^9 50% lethal doses (LD_{50}) per mg protein.

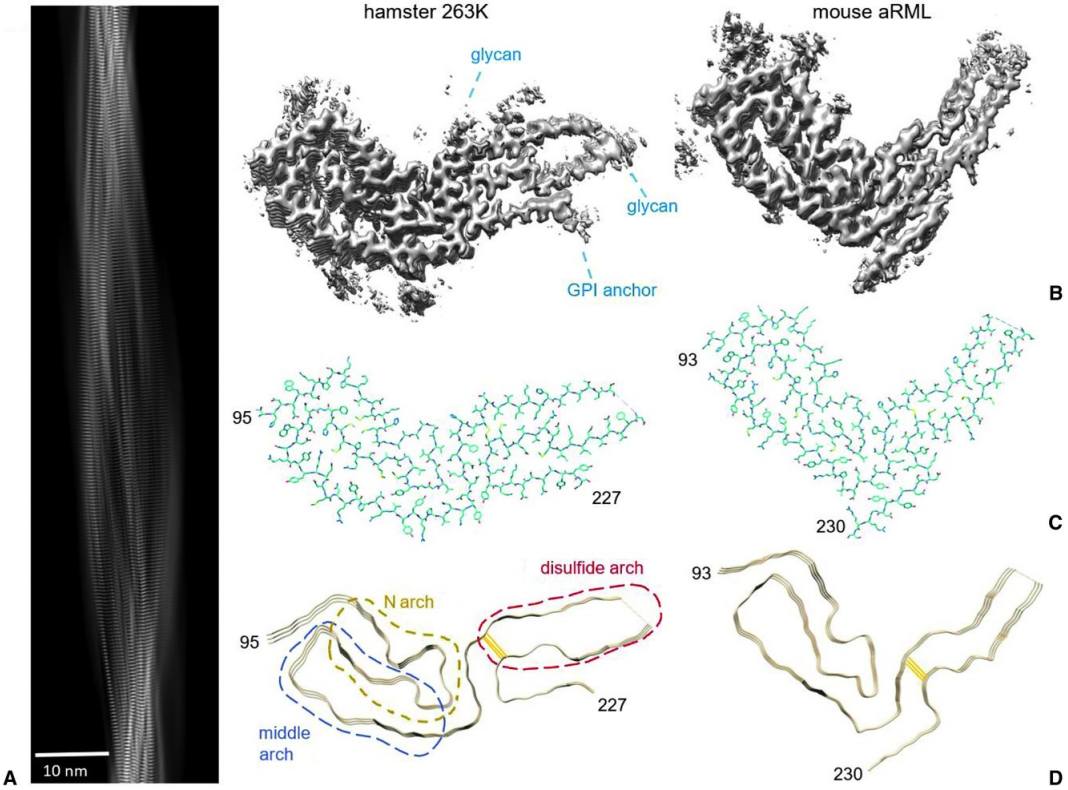

FIGURE 22.5 **Cryo-EM–based structures of two strains of brain-derived scrapie prion fibrils.**[178,212] **A**. Projection of hamster 263K fibril density map showing axial twist and cross-β striations running perpendicular to the fibril axis. **B**. Magnified end-on (cross-sectional) views density maps of hamster 263K and mouse aRML prion fibrils. The attachment sites of the mostly unresolved N-linked glycans and GPI anchor are indicated on the 263K map. **C**. Stick depictions of monomeric units of atomic models. **D**. Ribbon diagrams of stacks of three monomers with structural motifs circumscribed in the 263K model that are similar, but not identical, in aRML. *Dashed lines* indicate poorly resolved tips of the disulfide arches. (Panel **A** adapted from Figure 1E of Kraus A, Hoyt F, Schwartz CL, et al. High-resolution structure and strain comparison of infectious mammalian prions. *Mol Cell*. 2021;81(21):4540-4551. e6. Copyright © 2021 Elsevier, with permission.)

The core of the hamster 263K prion fibrils contains arches that partially overlap those seen in the synthetic PrP fibrils,[142,156,401] namely an N-proximal (N) arch spanning residues ~95-144 and a disulfide arch of residues ~170-229 (Fig. 22.5B-D).[212] A major difference is that the much larger 263K core contains both of these types of arches rather than just one or the other. Also, the detailed conformations of these arches in the 263K prion fibril differ markedly from those of the synthetic fibrils. A third major arch not seen in the synthetic protofilaments, named the middle arch (residues ~125-168), shares its N-terminal flank with the N arch. The tip of the middle arch forms a steric zipper with residues 96-100 to immobilize the N-terminus of the fibril core, the folding of which can strongly modulate infectivity.[286] Steric zippers are frequent features of amyloid fibrils[126] and can be important mediators of PrP fibril stability.[96] Whereas the synthetic fibrils have two-protofilament cross sections, the 263K fibril cross section is spanned by a single PrP monomer. The N-linked glycans and GPI anchors can be inferred to project outward from the core, but these posttranslational modifications are too heterogeneous to be resolved by cryo-EM.

The two additional near-atomic prion structures are of the highly infectious mouse-adapted RML scrapie strain in both its wild-type (wtRML)[253] and GPI-anchorless (aRML)[178] forms. The core structures of wtRML and aRML PrP^{Sc} are similar to each other but distinct from hamster 263K fibrils in both amino acid sequence (at eight positions within their ordered cores) and conformation (Fig. 22.5B-D). aRML differs additionally from 263K and wtRML in lacking GPI anchors, being severely underglycosylated, and accumulating predominantly in extracellular amyloid plaques rather than more diffuse membrane-associated deposits (Fig. 22.2A and B). Like 263K, aRML and wtRML prion fibrils have a PIRIBS architecture with N, middle, and disulfide arches, but the specific conformations and relative orientations of these arches and other structural features of these two prion strains differ markedly[178,253] (Fig. 22.5B-D).

PrP Fibril Structures and Transmissibility

Although the number of available high-resolution structures of PrP assemblies is limited, themes are emerging regarding features that influence relative prion infectivity: (1) Highly infectious PrP fibrils have larger tightly packed cores than marginally infectious, or noninfectious, fibrils.[163,213,286,360] Indeed, even small differences in the extent and structure of the core can profoundly affect infectivity[399] (Fig. 22.6). (2) Although glycans and GPI anchors strongly affect prion disease susceptibilities and phenotypes,[63,64,87,90,247,348,416] they are not required for infectivity[90,98,118,195,223,248,400] (see below). These high-resolution structures also allow the study of conversion of PrP^{C} to PrP^{Sc}, using molecular dynamic simulations, providing possible mechanisms for the species barrier between mice and hamsters (Fig. 22.7).

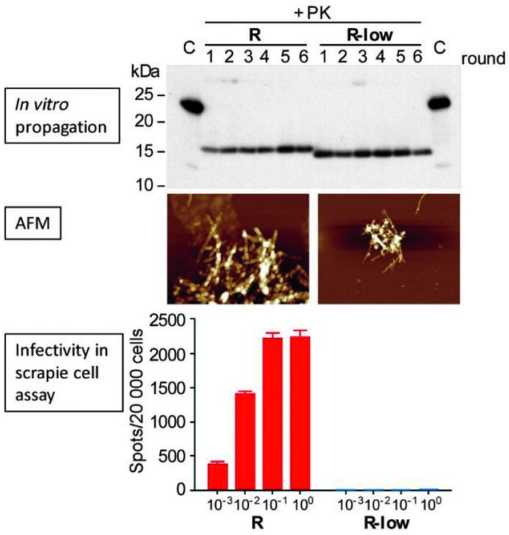

FIGURE 22.6 **Two conformers of murine PrP fibrils (R and R-low) formed and propagated under similar conditions *in vitro* differ markedly in infectivity.**[399] **Top**: The "R" and "R-low" fibrils maintain a ~1 kDa difference in their proteinase K (PK)-resistant cores through 6 rounds of *in vitro* amplification. "R" refers to the RNA cofactor that was included in the propagation of both types of fibrils. **Middle**: Atomic force microscopy (AFM) images of the "R" (*left*) and "R-low" (*right*) preparations indicate that both have fibrillar ultrastructures. **Bottom**: A live-cell–based assay of infectivity measured on across four 10-fold dilutions, indicating a difference in infectivity of at least 10 000-fold. (Reprinted from Figures 1A,C and 3B of Caughey B, Kraus A. Transmissibility versus Pathogenicity of Self-Propagating Protein Aggregates. *Viruses*. 2019;11(11):1044. http://creativecommons.org/licenses/by/4.0/. Adapted from Figure 2 of Wang F, Wang X, Orrú CD, et al. Self-propagating, protease-resistant, recombinant prion protein conformers with or without in vivo pathogenicity. *PLoS Pathog*. 2017;13(7):e1006491.)

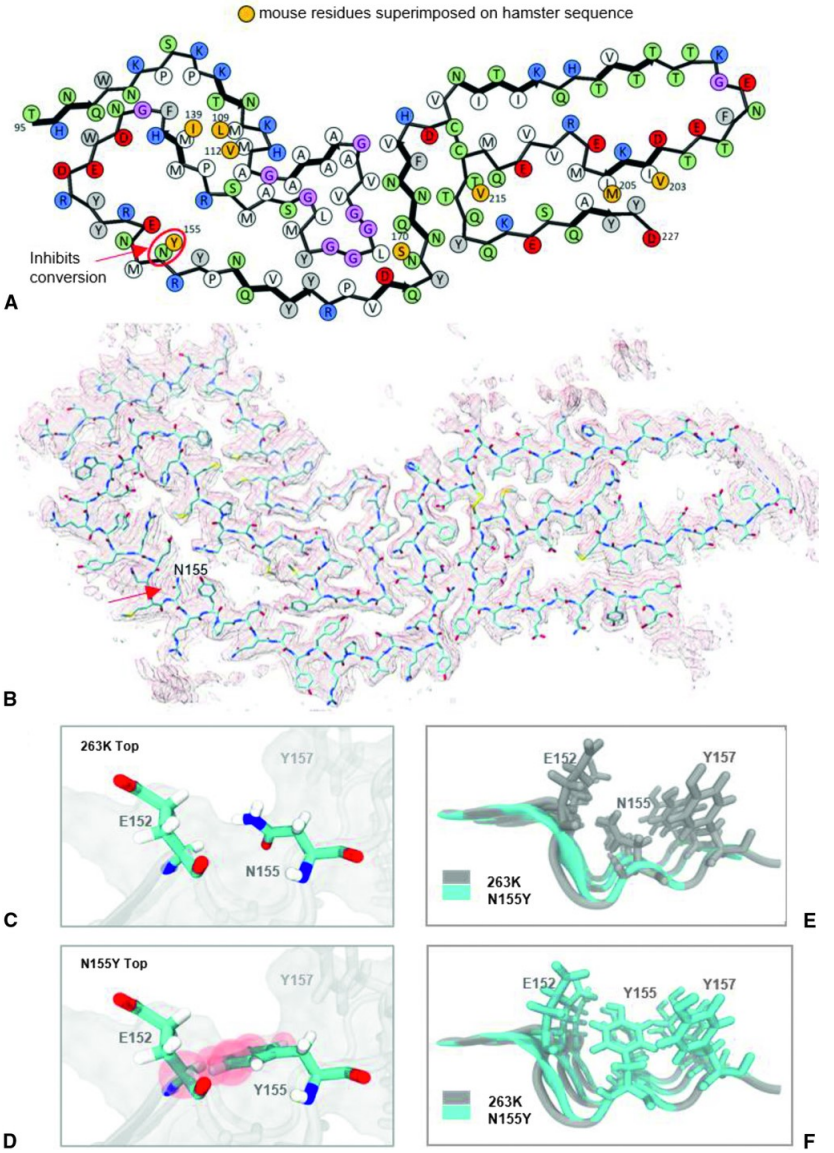

FIGURE 22.7 **Modeling of mechanism of the hamster-mouse transmission barrier**. **A**. Comparison of hamster and mouse PrP sequences in 263K prion cross section. Mouse residues (*orange*) are indicated next to the corresponding hamster residues. N155Y substitution in the hamster sequence impedes 263K-induced conversion.[310,346] **B**. PrP residues 95-227 threaded through a cross-sectional density map (mesh) with the *red arrow* indicating N155. **C**. Top view of the 263K structure at N155. **D**. Top view of 263K with N155Y mutation indicating steric clashes (*red translucent circles*). **E**. Top view of the 263K structure in *grey* illustrates the orientation of the N155, E152, and Y157 residues with deviations to accommodate the N155Y mutation shown in *turquoise*. **F**. Structural changes to alleviate the steric hindrance caused by incorporation of Y155 (*turquoise*). (Adapted from Figure S8A-H of Kraus A, Hoyt F, Schwartz CL, et al. High-resolution structure and strain comparison of infectious mammalian prions. *Mol Cell*. 2021;81(21):4540-4551. e6. Copyright © 2021 Elsevier, with permission.)

THE NORMAL, NONPRION ISOFORM OF PrP (PrP^C)

As the obligate precursor of PrP^{Sc}, PrP^C figures prominently in PrP prion disease pathogenesis. PrP^C and homologs thereof are found in many mammals and other vertebrates.[404] Although the overarching physiological function(s) of PrP^C remain unclear,[72,341,352] the structures from different species show a degree of conservation that suggests retention of a significant function through evolution.[313] Interactions of PrP^C with multiple ligands,[341,352] as well as effects on processes such as cellular differentiation, signaling, adhesion, and stress tolerance,[45] suggest a cell-surface scaffolding function that organizes and mediates a variety of physiological functions.[231]

Given that refolding of PrP^C molecules is fundamental to prion propagation, much attention has focused on their structures. Mammalian PrP^C molecules share the features summarized in Figure 22.1. The most abundant forms of PrP^C contain asparagine-linked glycans at one or both consensus glycosylation sites[170] as well as a GPI anchor[364] that targets PrP^C to lipid raft domains of membranes.[283] However, minor subsets of PrP^C lack glycans and/or GPI anchors.[363] PrP^C can be found in many cell types and tissues but is particularly abundant in neurons. As a glycoprotein, it is found along the secretory pathway, at the cell surface, and in endocytic and other membrane-bound transport vesicles (Fig. 22.1C).[78] PrP^C is also found in the inner mitochondrial membrane.[130] Cleavage near the C-terminus by ADAM 10 protease can release PrP^C molecules into extracellular spaces, a process that can be stimulated by events such as ligand binding.[232,376] An internal cleavage of PrP^C under physiological conditions can generate N- and C-terminal fragments, which can influence cellular functions.[65,103,227,252]

Although *Prnp* knockout mice are resistant to prion disease,[60] they are viable and healthy under controlled laboratory conditions.[61] Partially or wholly, PrP-deficient humans,[274,275] goats,[39] and cattle[329] are also generally healthy, suggesting that either PrP^C deficiency can be largely compensated by backup mechanisms or that PrP^C's function is not crucial for viability. However, there are studies indicating roles of PrP^C in many physiological processes such as circadian rhythm,[383] lymphocyte activation,[69] axonal myelination,[214] development,[254,365] neuronal signaling; synapse formation, neurite outgrowth,[8,340] cell adhesion,[343] metal ion homeostasis,[53] neuroprotection,[41,119] alcohol sensitivity,[306] and meditation of the neurotoxicity of oligomers of other proteins such as Aβ, α-synuclein, and tau.[40,72,104,133,220,231, 291,299,328,352,381] A note of caution is that some studies using PrP-deficient experimental models may be confounded by uncontrolled influences of flanking genes, overexpression, or ectopic expression.[121,366,369]

Prion Disease Pathogenesis

Given that organisms have quality control (proteostatic) mechanisms to counter the accumulation of abnormally folded proteins, a fundamental feature of proteinopathies is a kinetic race between aggregate replication/accumulation and clearance. Direct comparisons in prion-infected cell cultures have shown that PrP^{Sc} has a much slower turnover rate than PrP^C,[49,79] but studies have indicated that hosts have some ability to clear PrP^{Sc}.[309,336] Even so, with inoculation of a sufficient quantity of prions, for example, as little as few femtograms of PrP^{Sc}, the kinetic race is always won by prion replication, resulting in the demise of a susceptible host. This pathogenic process can be highly reproducible with death occurring months after inoculation and rodents of similar genetic background dying within days of each other. A primary factor controlling prion incubation periods is the level of PrP^C expression in the host. For example, whereas mice expressing wild-type levels of PrP^C can have scrapie incubation periods of <165 days when inoculated with a given dose and prion strain by a given route, hemizygous knockout mice can have incubation periods of 400-465 days when given the same challenge.[314]

Prion Growth

The detailed step-by-step mechanism of PrP^{Sc}-induced conversion of PrP^C to new PrP^{Sc} is unclear. Nonetheless, the respective conformations of PrP^C and PrP^{Sc} indicate that major events in this conversion process are the peeling of the first helix away from the other two helices, as well as a straightening of each of the helices into extended chains or loops that form intermolecular β-sheets and other linkages to the corresponding residues in the templating monomer at the ends of the fibrils (Fig. 22.8A).[212] It is not certain whether PrP^{Sc} interacts directly with individual PrP^C monomers or partially unfolded oligomers, but the net effect is that each incoming monomer usually assumes the conformation of the PrP^{Sc} template.[43,335,351,377] As the prion assembly grows, fragmentation must generate more prion particles *in vivo*[271] via a poorly understood mechanism.

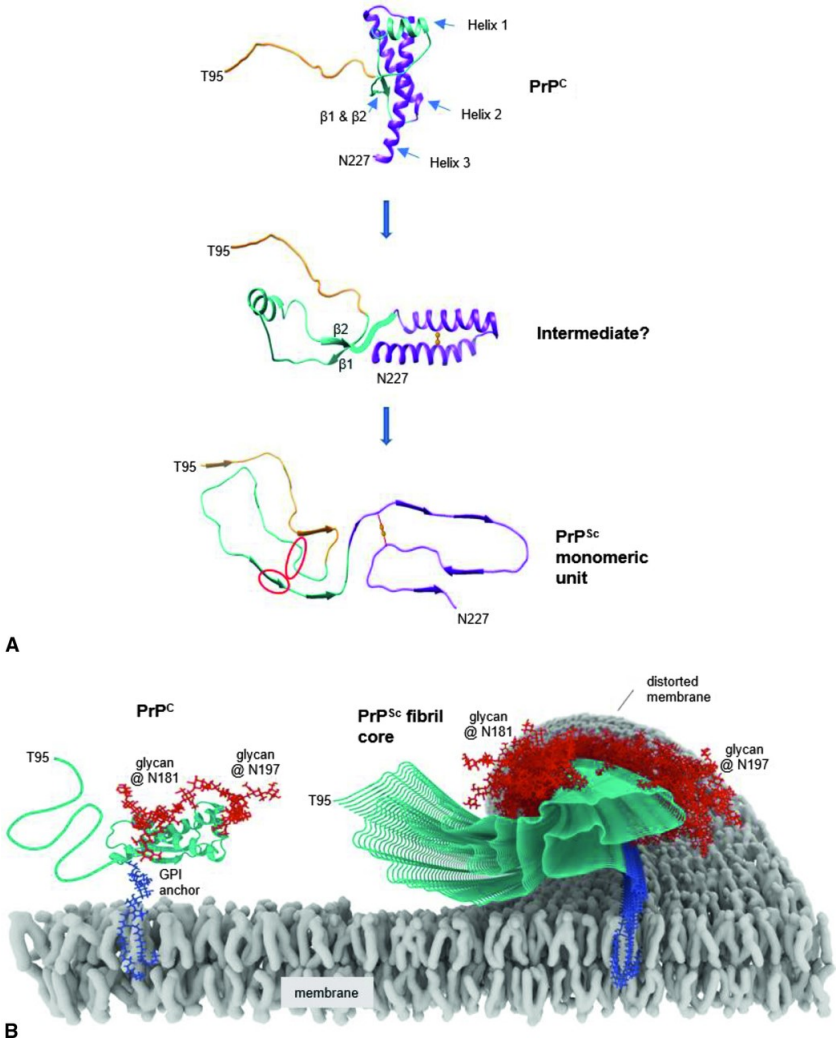

FIGURE 22.8 **Surmised conversion of PrP^C to PrP^{Sc} formation**. **A**. Hamster PrP residues 95-227 spanning the ordered core of the 263K prion fibril (*bottom*)[212] are depicted on top as PrP^C with the conformation of residues 125-227 as determined by NMR[182] and remaining residues 95-124 (*orange*) as an arbitrary flexible chain. A hypothetical intermediate in the middle illustrates the need for the β1-helix 1-β2 loop (*turquoise*) of PrP^C to separate from helices 2 and 3 (*purple*). The β1-β2 sheet of PrP^C must break to form intermolecular bonds in PrP^{Sc} (new positions encircled in *red*). **B**. Depictions of residues 95-231(*turquoise*) of PrP^C (monomer) and PrP^{Sc} (multimer) bound to a membrane with representative glycans (*red*) and GPI anchors (*blue*). The curl of the membrane (*gray*) is shown hypothetically to follow the axial twist of the GPI anchors. (Adapted from Figure 4 of Kraus A, Hoyt F, Schwartz CL, et al. High-resolution structure and strain comparison of infectious mammalian prions. *Mol Cell*. 2021;81(21):4540-4551. e6. Copyright © 2021 Elsevier, with permission.)

Prion Spreading Within Hosts

In sporadic CJD (sCJD), which represents a preponderance of human prion disease cases, the origin of the pathogenic PrP aggregation process is difficult to ascertain. In experimental animals, highly localized inoculations into sites as distal as the foot pad[155] have shown that prions can spread from remote areas into the brain.[33,155,243,352] Most studies have indicated that prion spreading occurs most efficiently via neural circuitry[199,349] and is dependent on levels of membrane-bound PrP^C in those neurons.[51,155,200] However, other, even hematogenous, routes also appear to be possible.[25,27,387] Nonneuronal spreading can also be important in, for example, the initial spread of infection from the gut to the peripheral nervous system.[70,243] In this scenario, key players in prion transport to the nervous system are follicular dendritic cells of secondary lymphoid organs. The extent to which other nonneuronal modes of spread might also be significant in ferrying prions to the brain is not clear. Chronic lymphocytic inflammation in the host can extend the tissue distribution of prions,[172] and *in vitro* studies indicate that the intercellular spread of prions and other proteopathic seeds can be enhanced by viral coinfections.[222,233] Even without experimental induction of systemic inflammation, prions have been detected in many tissues of infected hosts, including lymphoid tissues, skin, eyes, blood, and muscle[9,123,124,172,295-297,333] (see later sections); however, the apparent prion titers in such peripheral tissues are usually much lower than those of the brain.

Cellular and Molecular Pathogenesis

When neuronal cell lines are exposed to exogenous prions, they can internalize the prions into late endosomes and lysosomes and transport them along neuritic processes to points of contact with other cells (Fig. 22.9A).[245,309] The initial uptake has been reported to be facilitated by the presence of heparan sulfate on the cell surface,[176] but another study has suggested that sulfated glycosaminoglycans are dispensable for prion uptake.[417] The internalization pathways can vary with prion strain.[132] Establishment of persistent infection appears to involve transport from late endosomes or lysosomes into the endocytic recycling pathway.[256,421] In persistently infected cell cultures, PrP^{Sc} is formed posttranslationally from mature PrP^C molecules after they have been transported to the cell surface.[49,79] In most such cells, PrP^{Sc} can then be endocytosed and N-terminally truncated by endo-lysosomal proteases.[81,374] The initial site of conversion is either on the plasma membrane or in endocytic vesicles[50,81,159,256,422] and is most efficient when the two isoforms are in the same membrane.[29] Strikingly, nascent PrP^{Sc} can be visualized on the cell surface in patches, strings, or webs that resist endocytosis for hours and appear to be membrane-anchored PrP^{Sc} amyloids.[331,405] Spreading between cells[309] can be enhanced by cell-to-cell contact,[192] tunneling nanotubes,[160] and extracellular vesicles[394] (Fig. 22.9B and C).

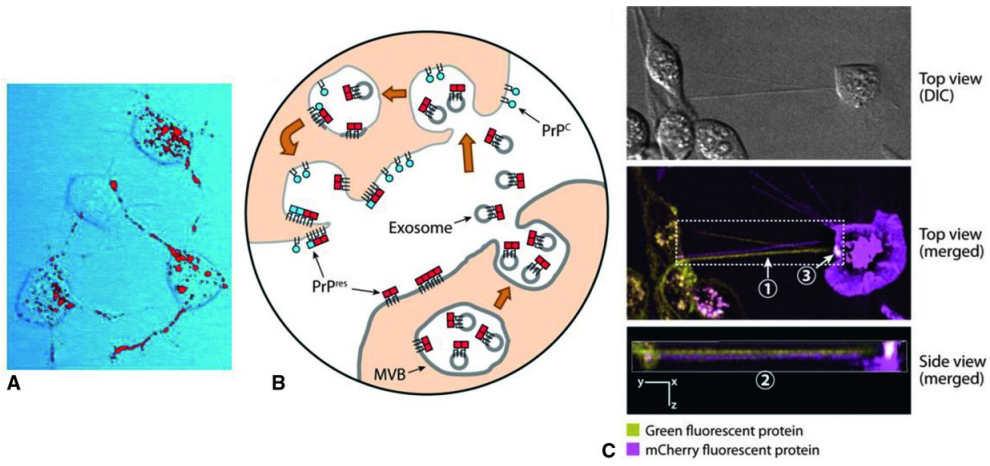

FIGURE 22.9 **Movement of prions within and between cells**. **A**. Pseudo-colored fluorescence microscopy image of neuronally derived SN56 tissue culture cells showing internalization and neuritic transport of exogenenously added fluorescently tagged PrP^{Sc} (*red*) to points of contact with other cells. **B**. Diagram of intercellular spread of PrP^{Sc} via exosomes, which form within multivesicular bodies (MVB) and can then be released into extracellular spaces and internalized by other cells. **C**. Differential interference contrast (DIC) and fluorescence images of threadlike tunneling nanotubes (*1* and *2*) between cocultured cells expressing GPI-anchored proteins tagged with either green fluorescent protein (GFP) or mCherry fluorescent protein. Number *3* points to a site where a tunneling nanotube from a GFP expressing cell has transferred a GFP-tagged protein onto a cell expressing a GPI-linked mCherry-tagged protein. (**A** is courtesy of Dr. Kil Sun Lee while at NIAID and B and C adapted with permission from Figure 7 of Caughey B, Baron GS, Chesebro B, et al. Getting a grip on prions: oligomers, amyloids, anchors and pathological membrane interactions. *Annu Rev Biochem*. 2009;78:177-204. Copyright © 2009 by Annual Reviews, http://www.annualreviews.org.)

In cultured cells that express various human genetic prion disease–linked PrP^C mutants, early misfolding and aggregation may in some cases

occur in the endoplasmic reticulum, along with incomplete maturation of glycans and delayed transport to the cell surface.[19,85,125] Such aberrant structures are nonetheless transported to post-ER components of the secretory pathway, where they are transferred to acidic lysosomal compartments for degradation.[19] A recent study has further described post-Golgi pathways for misfolded mutant PrP in neurons.[85] One pathway resembles the rapid ER stress-induced export (RESET) mechanism that is observed in nonneuronal cells in which the mutants are transported to lysosomes for immediate degradation. Another newly described pathway, dubbed *a*xonal *r*apid *e*ndosomal *s*orting and *t*ransport-dependent *a*ggregation (ARESTA), involves post-Golgi sorting to late endosomal structures called endoggresomes that are then subjected to anterograde transport into axons where they can fuse, aggregate, impair calcium transport, and hasten cell death.[85]

In the brain, nascent PrP^{Sc} has been detected near the outer basement membrane of blood vessels early after microinjection of scrapie brain homogenate (Fig. 22.10)[89] and then in neuronal plasma membranes and in both axons and dendrites.[158] Interestingly, injection of normal brain homogenate leads to dense aggregation of PrP^{C} near the needle track, but such aggregates are soon cleared.[89,402] These and multiple other studies are consistent with a role of PrP^{C} in response to tissue injuries and stresses.[264] Among the early deficits seen after prion infections is established in the brain are synaptic and mitochondrial dysfunction followed by axon terminal degeneration, dendritic atrophy, unfolded protein response, translational repression, and reactive gliosis.[66,113,139,157,187,188,255,280,373] Despite conflicting initial reports, microglial responses now appear to be protective against multiple strains of prions in mice.[66] Of likely relevance is a recent description of microglial networks picking up prionlike α-synuclein fibrils from neurons and either degrading them if they can, or, if not, passing them on to adjacent, less-burdened microglia.[342] Also protective in prion disease is the presence of SARM1, a factor that under other circumstances can promote axonal degeneration.[427] Ultimately, prion infections can lead to cell death, autophagy, and spongiform change.[354,414] The sequence of PrP^{Sc} accumulation and its neurotoxic effects has been particularly well visualized in the retinas of infected mice where photoreceptor degeneration begins with PrP^{Sc} deposition at the cilia and ribbon synapses of cones[370] (Fig. 22.11).

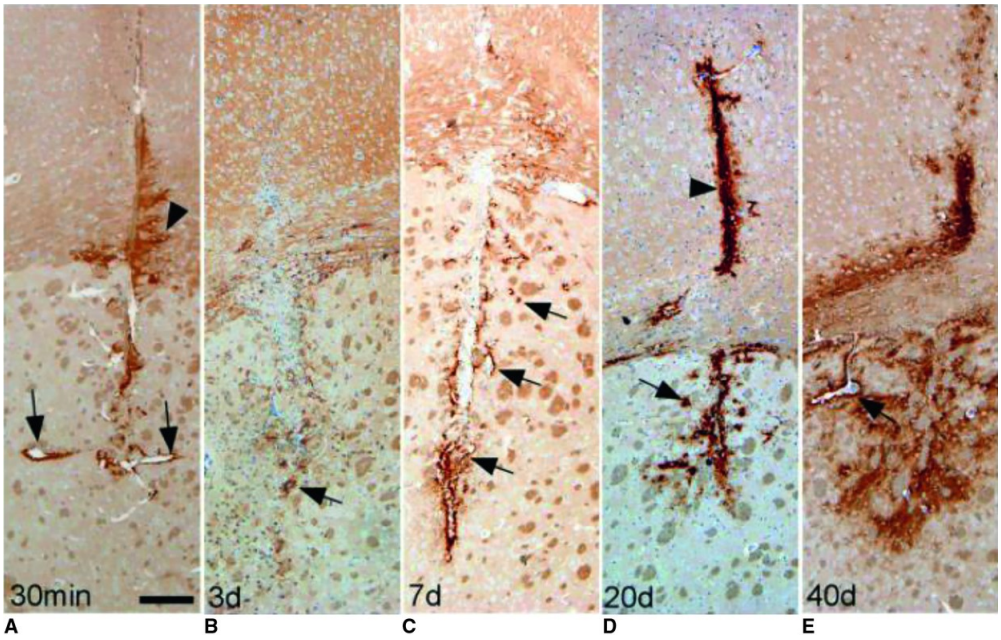

FIGURE 22.10 **Time course of immunostaining of PrP^{d} (darker brown against lighter, more diffuse staining of PrP^{C}) after intracerebral microinjection of scrapie strain 22L into wild-type mice**. **A**. At 30 minutes, staining of the inoculum is seen around blood vessels (*arrows*) in the striatum and along the needle track in the corpus callosum and cortex (*arrowhead*). **B**. Reduced staining at 3 days suggests initial clearance of inoculum. *Arrow* indicates blood vessel–associated staining. **C-E**. Subsequent increase in staining from 7 to 40 days. *Arrows* and *arrowheads* mark blood vessel and wound-associated staining respectively. (Reprinted from Figure 2 of Chesebro B, Striebel J, Rangel A, et al. Early generation of new PrPSc on blood vessels after brain microinjection of scrapie in mice. *MBio*. 2015;6(5):e01419-15. Copyright © 2015 Chesebro et al.)

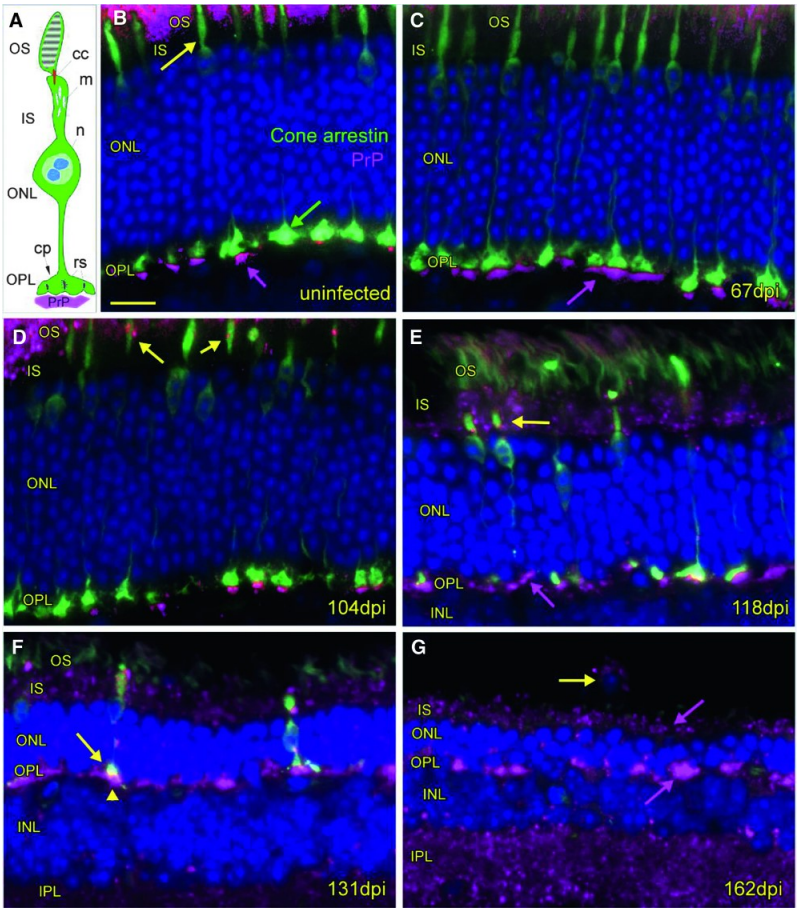

FIGURE 22.11 **Time course of damage in retinas of scrapie-infected mice**. **A**. Cartoon of cone photoreceptor (*green*) within retina. PrP (*pink*) is located below cone pedicle (cp) containing ribbon synapses (rs). OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer, cc; connecting cilium, m; mitochondria, n; nucleus, cp; cone pedicle, rs; ribbon synapse. **B**. Cone photoreceptor inner segments (*yellow arrow*) and pedicles (*green arrow*) are stained with cone arrestin (*green*) in uninfected mouse. *Pink arrows* indicate PrP. **C**. At 67 days post infection (dpi), staining pattern is similar to uninfected mouse. **D**. At 104 dpi, small irregular PrP^{d} deposits associated with cones become apparent (*yellow arrows*). **E**. At 118 dpi, PrP^{d} accumulation is more extensive in IS and OPL, cone numbers are reduced (*green*), cone pedicles have disappeared from the OPL, and remaining cones are dystrophic (*yellow arrow*). **F**. At 131 dpi, few cones remain, and pedicles (*yellow arrow*) are smaller and associated with PrP^{d} (*arrowhead*). **G**. Cones are no longer detected and the ONL is thinner, indicating loss of photoreceptors. *Yellow arrow* marks a macrophage or microglial cell containing PrP^{d}. *Bright pink* in OS is autofluorescence. Scale bar in **A** = 20 µm. (Adapted from Figure 5A and 6 of Striebel JF, Race B, Leung JM, et al. Prion-induced photoreceptor degeneration begins with misfolded prion protein accumulation in cones at two distinct sites: cilia and ribbon synapses. *Acta Neuropathol Commun*. 2021;9(1):17. http://creativecommons.org/licenses/by/4.0/.)

Mechanisms of PrP^{Sc} Toxicity

The mechanisms of PrP^{Sc} toxicity, whether direct or indirect, are not well understood. Several aspects of neuronal proteostasis can be perturbed by prion infections in ways that might be deleterious to neurons.[352] These include the disturbances of the ubiquitin/proteasome system,[263] autophagy and lysosomal pathways,[1,420] the unfolded protein response,[280] stress protective signaling,[323] and mitochondrial function.[5,95,130,225] Direct association of PrP^{Sc} with membranes results in deformed membrane structures (Fig. 22.2C-G).[73,185,186,212] Such deformities seem likely to affect a variety of cellular functions, including those involving intracellular transport[127,128] and neuronal receptor signaling.[92,345] Major aspects of PrP^{Sc} toxicity also appear to be mediated through interactions with cell surface PrP^{C}.[51,87,221,249] Moreover, as infectivity plateaus prior to onset of symptomatic prion disease, PrP^{C} levels drop along with an increase in oligomeric forms of PrP^{Sc} that may be particularly neurotoxic.[261] However, loss of PrP^{C} function does not appear to be the sole reason for prion disease pathology because hosts with depleted PrP^{C} alone do not develop prion disease.[61,249,326]

Implications of GPI-Anchoring and Glycans of PrP^{Sc}

For at least some prion strains, the GPI anchors and N-linked glycans do not appear to markedly affect the core β-sheet structure,[28,178,253] and serial passages from wild-type mice through anchorless PrP transgenic mice and back again do not alter the fundamental prion strain characteristics.[87] However, the outward display of the N-linked glycans and GPI anchors, when present, is likely to have major implications in pathogenesis.[212] Comparison of prion strains in mice expressing PrP^{C} with or without GPI-anchors and/or N-linked glycans have shown profound phenotypic differences.[64,87,90,200,247,320,348,416] For example, as noted above, PrP^{d} in anchorless mice is found not in diffuse, cell-associated deposits, but in extracellular amyloid plaques (Fig. 22.2B) reminiscent of those seen in human Gerstmann-Sträussler-Scheinker

syndrome (GSS)[149] and kuru.[230]

The cryo-EM structure of hamster strain 263K suggests that the presence of, and variations in, glycans and GPI anchor modifications could strongly modulate the interactions of prions with their tissue environments. As depicted in Figure 22.8B, the glycans and GPI-mediated membrane tethering should severely restrict access of other macromolecules to at least the C-terminal half of the 263K fibril core whether they might be cofactors for prion growth or proteostatic factors involved in prion recognition and clearance. Also, several pathognomonic PrP^{d}-associated membrane distortions have been described in prion-diseased brains that might be attributable to tight tethering of membranes to PrP^{Sc} fibrils via the linear array of GPI anchors as they follow the twist of the fibril (Fig. 22.8B),[212] including invaginations, protrusions, and spiral membrane inclusions (Figs. 22.1C and 22.2D-G).[73,185,228] Membrane associations might also be expected to help mediate cell-to-cell spreading of prions via membranous structures such as exosomes and tunneling nanotubes (Fig. 22.9B and C).[73,160,394] Nonetheless, although disease phenotypes are affected dramatically by GPI anchors and glycans, PrP^{Sc} accumulation can be fatal and highly transmissible either with or without these posttranslational modifications on either PrP^{C} or PrP^{Sc}.[87,90,200,320]

Human Prion Diseases: Epidemiology and History

Human prion diseases are rare TSEs affecting 0.3-1.7 people per million population per year, with a peak incidence in the seventh decade of life.[217,389] They can be classified as sporadic, genetic, or acquired. Sporadic cases represent 85% of all cases, with the remaining 15% being genetic and <1% acquired.

The history of human prion diseases is one of misclassification and serendipity. The most common type is sporadic Creutzfeldt-Jakob disease (sCJD). The name derives from one case report by Hans Gerhard Creutzfeldt and five case reports by Alfons Maria Jakob in 1920-1923, that described patients with rapidly progressive motor, speech, and cognitive deficits that were ultimately fatal.[194] Of note, it was Jakob who proposed that Creutzfeldt's case could be one of the same syndromes he reported, but a more recent analysis of brain samples from these cases has revealed that Creutzfeldt's case was actually not a prion disease, nor were all of Jakob's initial cases.[194] Nonetheless, the eponym remains and is used to refer to the most common types of human prion disease.

Sporadic Human Prion Diseases (85%)

Patients with CJD typically present with a rapidly progressive neurodegenerative syndrome, but there is considerable variability in the presence and timing of symptoms, including ataxia, visual disturbances, parkinsonism, dementia, and myoclonus. Even disease duration can vary from weeks to a few years.

Before the identification of PrP, distinct clinical presentations of CJD were categorized by phenotype. The Heidenhain variant referred to patients with prominent visual symptoms, including hallucinations, distortions, cortical blindness, and/or Anton syndrome,[171] and the Brownell-Oppenheimer variant was used to describe patients with predominantly ataxia.[57] Today, six subtypes are recognized, based on patient *PRNP* codon 129 status, PrP electrophoretic mobilities after proteinase K digestion, and neuropathological patterns of PrP deposition (diffuse synaptic or more plaquelike) and spongiform change[303,358,425] (Fig. 22.12, Table 22.2).

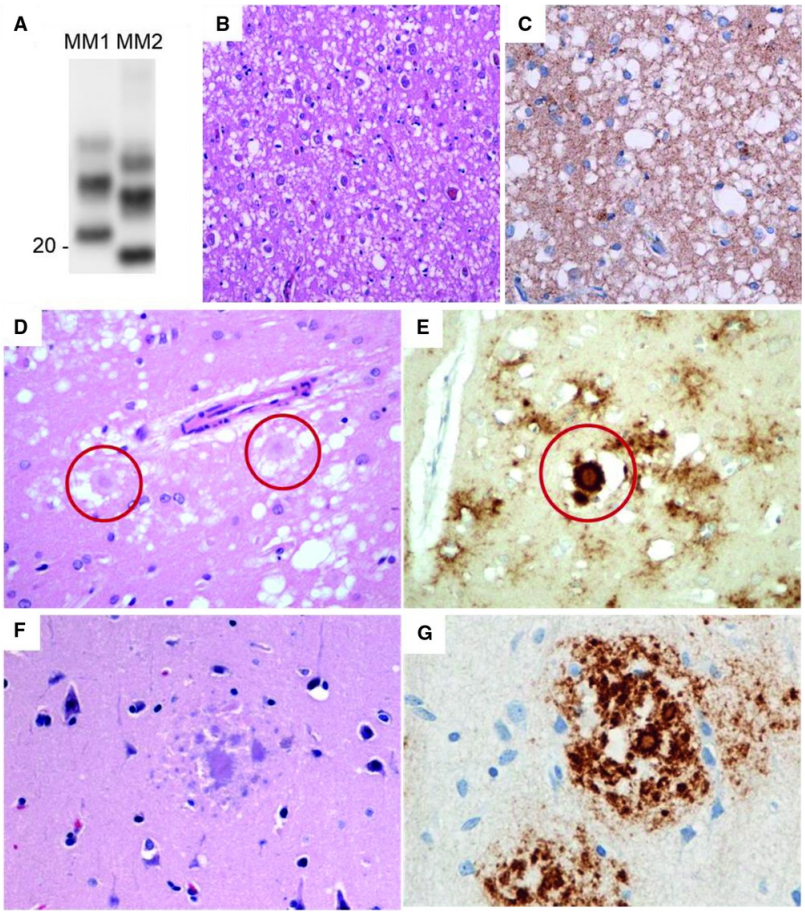

FIGURE 22.12 **Pathological findings in human prion disease**. **A**. Immunoblot comparing PK-digested banding profiles of CJD subtypes MM1 and MM2 (3F4 antibody). Type 1 has a lower band of 21 kDa; type 2 is 19 kDa. **B**. Sporadic Creutzfeldt-Jakob disease (sCJD) brain: Hematoxylin and eosin stain showing typical spongiform change. **C**. sCJD brain: Immunostaining for prion protein (12F10 antibody) showing diffuse synaptic pattern (*brown*). **D**. Variant Creutzfeldt-Jakob disease (vCJD): Periodic acid-Schiff stain showing typical florid plaque surrounded by vacuoles (*red circles*). **E**. vCJD: Immunostaining for prion protein (12F10 antibody) showing a florid plaque (*red circle*). **F**. GSS: Hematoxylin and eosin stain showing a typical multicentric plaque. **G**. GSS: Immunostaining for prion protein (12F10 antibody) showing multicentric plaques. (Pathology images courtesy of Dr. Gerard Jansen, University of Ottawa. Panels B-G adapted from Figure 1A-F of Sim VL. Prion diseases. In: Jackson AC, ed. *Viral Infections of the Human Nervous System*. Springer; 2013:371-401.358)

TABLE 22.2 Subtypes of sporadic Creutzfeldt-Jakob disease

Molecular Type[a]	Onset: Years (Range)	Duration: Months (Range)	Clinical Presentation	Diagnostics	Neuropathology
M(M/V)1 MM1 and MV1 are considered the same subtype	68 (31-86)	4 (1-24)	Rapid dementia Myoclonus 50% ataxia at onset 30% visual impairment	MRI: basal ganglia and cortex EEG positive	Neocortex (predominantly occipital), striatum, thalamus, and cerebellum; "synaptic" PrP
VV2	64 (40-83)	6.5 (3-18)	Ataxia at onset Dementia later	MRI: basal ganglia and thalamus EEG negative	Prominent in cerebellum and subcortical, late cortical involvement; plaquelike and perineuronal PrP
MV2K (K for Kuru plaques)	65 (36-83)	17 (4-48)	Dementia Ataxia Extrapyramidal signs	MRI: basal ganglia and thalamus EEG negative	Amyloid kuru plaques in the cerebellum; plaquelike focal PrP deposits
MM2C (C for cortical)	64 (49-77)	16 (9-36)	Dementia Myoclonus Pyramidal signs Less frequent ataxia	MRI: cortex EEG negative	Spongiosis with large confluent vacuoles in cortex; perivacuolar PrP
MM2T (T for thalamus) May be sporadic FI	52 (26-71)	16 (8-36)	Psychiatric features Depression Psychosis Parkinsonism	MRI: thalamic gliosis EEG negative	Atrophy of the thalamus and inferior olive, focal spongiosis limited to the cerebral cortex
VV1	39 (24-49)	15 (14-16)	Dementia at onset Late ataxia Late extrapyramidal	MRI: cortex EEG negative	Severe pathology in the cerebral cortex and striatum with sparing of brainstem nuclei and cerebellum

Subtypes are listed in order of decreasing frequency.

[a]Polymorphism at codon 129 of the *PRNP* gene (M, methionine or V, valine) as well as the PrP^{Res} classification (1 or 2) with additional pathological phenotypes as defined in parentheses. MRI findings specifically refer to typical areas of hyperintensity on FLAIR (fluid-attenuated inversion recovery) or DWI (diffusion-weighted imaging). A positive or negative EEG refers to the presence or absence of generalized discharges. See section on diagnosis for more details.

Adapted from Table 1 by permission from Springer: Sim VL. Prion diseases. In: Jackson AC, ed. *Viral Infections of the Human Nervous System*. Springer; 2013:371-401. Copyright © 2013 Springer Basel; and adapted from Table 9.3 of Zerr I, Parchi P. Sporadic Creutzfeldt-Jakob disease. *Handb Clin Neurol*. 2018;153:155-174. Copyright © 2018 Elsevier, with permission.

Factors Influencing Creutzfeldt-Jakob Disease Susceptibility and Phenotype

PRNP Gene Polymorphisms

In humans, *PRNP* polymorphisms at codons 127, 129, and 219 are genetic risk factors or modifiers of disease phenotype[267] (Fig. 22.13). The polymorphism at codon 129 encodes either methionine (M) or valine (V), with homozygosity of the M allele being a susceptibility factor for variant CJD and homozygosity of either allele enhancing risk of developing sporadic CJD.[268,302] Heterozygosity is associated with longer survival time.[308]

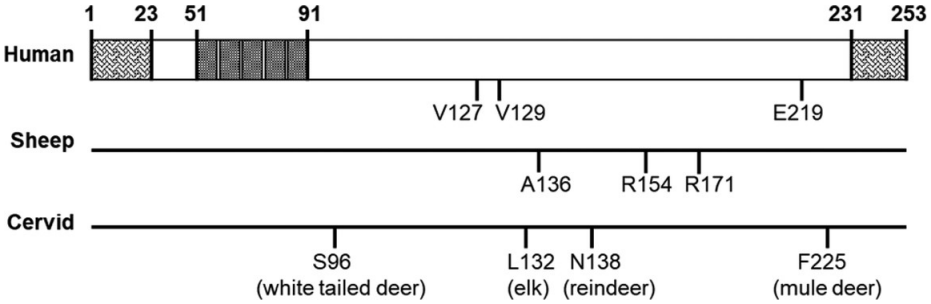

FIGURE 22.13 ***PRNP* polymorphisms known to modify disease in humans and other mammals**. Polymorphisms with resistance or protective effects are shown. (Adapted from Figure 76.11 of Prusiner SB. Prions. In: Knipe DM, Howley P, eds. *Fields Virology*. Vol 2. 6th ed. Wolters Kluwer Health/Lippincott Williams & Wilkins; 2013.)

The geographically localized variant, V127, is associated with resistance to kuru in New Guinea[269] and can render transgenic mice expressing human PrP V127 completely resistant to multiple prion strains.[17] The E219K polymorphism found in Japan is likely to be protective against sCJD.[350]

Non-PRNP Single Nucleotide Polymorphisms

A recent genome-wide association study identified two non-*PRNP* loci associated with increased risk of sCJD, specifically missense mutations in GAL3ST1 and a variant causing increased expression of STX6.[191] GAL3ST1 encodes galactose-3-*O*-sulfotransferase 1, an enzyme involved in sphingolipid metabolism, whereas STX6 encodes syntaxin-6, which is involved in endosomal recycling. The increased risk is modest at best, and given the rarity of CJD, the presence of two copies of both alleles only confers a lifetime risk of CJD of 1 in 3000.[390] Nevertheless, the discovery of these associations has opened new possibilities for studying disease pathogenesis and therapeutic design.

Other Sporadic Forms of Human Prion Disease

Variably Protease-Sensitive Prionopathy

A newer form of sporadic prion disease, variably protease-sensitive prionopathy (VPSPr), was identified in 2008[143] and is thought to account for 0.7%-1.7% of all sporadic prion diseases.[288] It fulfills the criteria of a prion disease in that it has been transmitted to animals.[288] Interestingly, while all codon 129 genotypes can be affected, the majority (65%) are VV, unlike sCJD, where the majority are MM. The phenotype involves more psychiatric presentations and speech/language impairment, before cognitive decline, and the neuropathology includes spongiform change and PrP amyloid miniplaques. The name derives from the atypical electrophoretic profile of five to seven PrP bands that are variably protease-resistant.[288] Diagnosis requires a high level of suspicion, because the MRI is usually normal, electroencephalography (EEG) is rarely abnormal, and often patients are older with longer disease durations, often 2 years. VPSPr has phenotypic and biochemical similarities to the V180I PrP gene mutation,[288] suggesting that it may reflect the sporadic form of this inherited disease (see Genetic Prion Diseases below).

Sporadic Fatal Insomnia

A thalamic form of sCJD, specifically categorized as MM2T, may represent a sporadic form of fatal insomnia (sFI), but sFI has more heterogeneity. For example, insomnia, while present in 29% of sFI patients at presentation, is a less prominent finding than in fatal familial insomnia (FFI), and atypical parkinsonism is more often present in sFI than in FFI.[110,279,316]

Genetic Prion Diseases (15%)

For any patient presenting with prion disease symptoms, it is prudent to perform *PRNP* sequencing, as the clinical presentation of sporadic cases and genetic forms can overlap. Furthermore, patients with heritable forms of prion disease may not always have a family history of the same.[209] Mutations include deletions, insertions, missense, nonsense, and splice site variants (Fig. 22.14). To date, all inherited prion diseases are associated with mutations in the *PRNP* gene itself, but some mutations are less penetrant.[275]

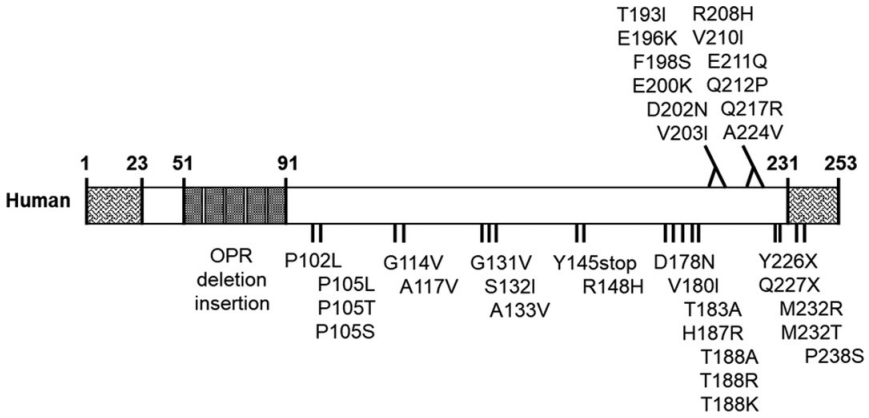

FIGURE 22.14 **Human *PRNP* mutations associated with prion disease**.

Historically, patients with inherited prion disease were categorized based on three clinical/neuropathological phenotypes: fatal familial insomnia (FFI), Gerstmann-Sträussler-Scheinker syndrome (GSS), and familial CJD. As we learn more about the PrP features underlying these diseases, and their overlapping clinical presentations, it may become more appropriate to classify these diseases based on their neuropathological profiles, with thalamic degeneration and PrP amyloidosis replacing FFI and GSS, respectively.

Thalamic Degeneration (Fatal Familial Insomnia)

In 1986, Lugaresi et al. first coined the phrase “fatal familial insomnia (FFI)” to describe a heritable neurodegenerative syndrome of progressive sleep disturbance, autonomic changes including pyrexia and diaphoresis, tremor, dysarthria, ataxia, myoclonus with cognitive decline progressing to akinetic mutism within a year of onset.[239] Pathological findings revealed severe atrophy of the thalamus, without marked spongiform change or amyloidosis. The association with a mutation in *PRNP* was identified in 1992.[251,270] In 1995, FFI was proven to be a transmissible prion disease.[101,375]

The *PRNP* mutation that gives rise to the FFI is D178N when it occurs *in cis* with a methionine at codon 129. Interestingly, if D178N occurs *in cis* with valine at codon 129, a phenotype of familial CJD occurs, which is indistinguishable from sporadic CJD. In patients who are heterozygous for the codon 129 allele, disease duration, clinical phenotype, and neuropathology are modified. 129MM patients have a shorter disease duration (11 ± 4 months) compared with 129MV patients (23 ± 9 months).[144] Homozygous patients typically present with sleep disturbances, with more prominent myoclonus, autonomic dysfunction, spatial disorientation, hallucinations, and weight loss, whereas heterozygotes often have early visual symptoms and ataxia, followed by dysarthria, seizures, and bulbar symptoms.[144,210]

Diagnosing FFI is challenging. MRI is less often abnormal, possibly because there is less spongiform change or PrP^d burden, and EEG reveals loss of sleep architecture but not the classic periodic discharges.[109] The real time-quaking-induced conversion (RT-QuIC) assay, which has high sensitivity and specificity for many types of human prion disease (see below), also has reduced sensitivity for FFI, reaching only 83% sensitivity at best.[339] Fluorodeoxyglucose positron emission tomography (FDG-PET) or single photon emission computed tomography (SPECT) may be helpful, in that it can reveal hypometabolism in the thalamus that spreads to other areas as the disease progresses,[298] but ultimately, the proof is the detection of the *PRNP* mutation.

PrP Amyloidosis (Gerstmann-Sträussler-Scheinker)

The history behind GSS is more complicated.[150] It was initially identified as a heritable neurodegenerative syndrome in an Austrian family in 1912,[145] where patients presented at a younger age (early 50s) with early ataxia, late cognitive decline, and often pyramidal and extrapyramidal features. The progression of disease was also much longer, often lasting 6 years. All patients had a characteristic neuropathology with multicentric amyloid plaques. Subsequent studies have identified these plaques as being composed of PrP^d.[151] GSS has also been shown to be transmissible to mice expressing human PrP with the P102L mutation.[16]

Over the following decades, at least 24 mutations in *PRNP* have been linked with GSS neuropathologies of cerebral amyloidosis (Fig. 22.14),[150] with the majority having P102L *in cis* with methionine at codon 129, as seen in the original GSS kindreds.[179] It is really the pathology that defines this group of cerebral amyloidoses more than the clinical phenotypes, because unlike FFI, GSS phenotypes overlap with different mutations, and there are now four recognized clinical phenotypes associated with the P102L mutation alone, including the typical GSS described originally, GSS with areflexia and paresthesia, pure dementia GSS, and CJD-like GSS.[379] At least some of this variability appears to relate to the relative levels of PrP^d generated from the mutant vs wild-type PrP allele in a given individual.[301,307]

A separate type of prion amyloidosis has also been identified as a cerebral amyloid angiopathy (CAA) in which PrP amyloid accumulates in the cerebral blood vessels. This is often associated with nonsense mutations causing truncated versions of PrP and can coexist with tau aggregates.[150]

In keeping with the distinct pathologies of these cerebral amyloidoses, a neuropathological classification has been proposed: (1) parenchymal PrP amyloid and prominent spongiform changes; (2) parenchymal PrP amyloid and minimal or no spongiform changes; (3) parenchymal PrP amyloid coexisting with severe tau neurofibrillary pathology, and absent or minimal spongiform changes; and (4) vascular PrP-CAA coexisting with severe neurofibrillary pathology.[150]

Systemically, there are also PrP amyloidoses that affect the gut and peripheral nervous system, causing syndromes associated with diarrhea and peripheral or autonomic neuropathy. To date, these have been linked to the nonsense mutations Q160X[136] and Y163X.[266]

Implications of Genetic Prion Diseases

The detection of a mutation in *PRNP* has implications for diagnosis, genetic counseling, and access to surgical procedures for asymptomatic carriers. Several mutations are considered 100% penetrant, including E200K, which causes genetic CJD; A117V and P102L, which cause GSS; and D178N, which causes either FFI if *in cis* with methionine at codon 129, or genetic CJD if *in cis* with valine at codon 129. However, sometimes patients with progressive dementia have *PRNP* mutations that are not causal or have low penetrance, so care must be taken before counseling family members of patients. For example, the penetrance of M232R, V180I, and V210I are low, with lifetime risks estimated to be 0.1%, 1%, and 10%, respectively.[275] Interestingly, the mutation sometimes influences the clinical phenotype; patients with E200K and V210I present similarly, with symptoms indistinguishable from sporadic CJD,[426] whereas V180I is associated with a later onset of slowly progressive dementia without many of the other motor or visual manifestations seen in CJD.[317]

Another important feature of genetic prion diseases is that the age of onset can vary greatly and cannot be predicted by age of onset in family members.[276] This makes it challenging both to counsel patients and to recruit patients into prophylactic treatment trials. In the future, predictive biomarkers, such as neurofilament light chain levels,[382] may help identify patients who are close to the clinical stage of disease.

Patients with a risk of inheriting a *PRNP* mutation, or who already know they are an asymptomatic carrier, can face challenges in access to health care. No cases of iatrogenic transmission from asymptomatic genetic carriers of *PRNP* mutations have been reported, so the risk remains theoretical. Tissues considered to have high infectivity are the same as those seen in sporadic CJD (Table 22.3). Most guidelines recommend that any surgical procedure involving higher-risk tissues requires that equipment go through additional sterilization procedures and that single-use tools be used whenever possible. Routine reprocessing procedures can be used for surgeries involving low-risk tissues. No cases of disease transmission by blood transfusion from sporadic or genetic CJD patients have been identified, but patients with a family history of CJD or a genetic carrier of CJD are generally not accepted as blood donors.

TABLE 22.3 Sporadic Creutzfeldt-Jakob tissue infectivity

Infectivity Titer	Tissue/Secretion
High	Brain, spinal cord, dura mater, pituitary gland, posterior segment of the eye (posterior hyaloid membrane, retina, retinal pigment epithelium, choroid, subretinal fluid, optic nerve)[56]
Low (case transmission to human/partial transmission to primate)	Cornea (1 definite and 1 probable iatrogenic human case following transplant) Cerebrospinal fluid, spleen, lymph nodes, lung, liver, kidney (partial transmission in primate studies[56])
Low (transmission to TgHuPrP mice)	Nasal olfactory mucosa,[325] blood,[124] bone marrow,[180] skin,[297] skeletal muscle, adrenal glands, thyroid, pancreas, salivary gland, heart, thymus, tonsil[123]
Detected only with ultrasensitive tests like RT-QuIC or PMCA	Blood vessels, peripheral nerves, urine,[240] placenta,[241] anterior parts of eye[295]
Below detection limits	Sweat, tears, nasal mucous, gingival tissue, feces, semen, GI tract, ovaries, uterus, adipose, bone[56]

Compilation of data based on iatrogenic human cases, animal transmission, and ultra-sensitive PrP^{Sc} detection studies.

RT-QuIC, real-time quaking–induced conversion; PMCA, protein misfolding cyclic amplification; TgHuPrP mice, transgenic mice expressing only human PrP.

Acquired Human Prion Diseases

Less than 1% of human prion disease cases are acquired. They can be iatrogenic or result from zoonotic exposure. In general, the incidence of acquired prion disease has decreased with the awareness of high-risk tissues (Table 22.3), improved surgical protocols, the cessation of using cadavers as source material for growth hormone and dura mater, and the decline in BSE, which caused variant CJD (vCJD) in humans.

Iatrogenic Cases

Patients have acquired CJD after exposure to corneal transplant, stereotactic EEG electrodes, neurosurgical instruments, cadaveric dura mater, pituitary-derived growth hormone, and blood transfusion from vCJD patients.[42,55] Of these, dura mater and human growth hormone recipients make up the vast majority, with at least 228 and 226 cases, respectively (Table 22.4). Incubation periods have ranged from 1 to 42 years after exposure.[55]

TABLE 22.4 Iatrogenic CJD

Source	Number of Cases	Incubation: Years (Range)
Dura mater graft	228	12 (1.3-30)
Neurosurgical instruments	4	1.4 (1-2.3)
Stereotactic EEG needles	2	1.3, 1.7
Cornea	2 (1 definite after graft; 1 probable after keratoplasty)	1.5, 27
Growth hormone	226	17 (5-42)
Gonadotropin	4	13.5 (12-16)
Packed red blood cells	3 (1 other died prior to symptom onset)	6.5, 7.8, 8.3

Adapted from Table 2 of Brown P, Brandel JP, Sato T, et al. Iatrogenic Creutzfeldt-Jakob disease, final assessment. *Emerg Infect Dis*. 2012;18(6):901-907.

A causal relationship between prior non-CNS surgery and risk of CJD has not been proven, but the quality of evidence available for assessing these associations is low, with prolonged incubation periods complicating associations and statistical significance depending largely on how the control group is selected.[116,237] In some studies, the timing of procedures may overlap symptom onset, and the majority of studies have found associations between procedures such as lumbar punctures, which are often done to investigate early clinical symptoms of CJD, and insertion of gastrostomy tubes, which are often provided to patients with inability to swallow, a finding that usually develops in later CJD.[112] In these situations, the link is clearly not causal as the procedures occur after symptom onset.

There has also been concern about eye surgeries, given the two cases of transmission via corneal transplant and the fact that the posterior component of the eye is considered high titer (Table 22.3). A confounding factor is the fact that the age of onset for CJD overlaps with the age of developing cataracts, and CJD patients may first present with visual symptoms to an ophthalmologist. Cataract surgery, which involves the anterior compartment of the eye, is not infrequently performed on CJD patients early in disease course. Studies assessing the risk of eye surgery on developing CJD are conflicting,[102,332,392] although separating out ophthalmic surgeries involving retina or optic nerve revealed a small association with CJD patients in one analysis.[115]

In the end, while iatrogenesis is difficult to prove, it is important to acknowledge that prions do exist in peripheral tissues of patients with sCJD (Table 22.3). Since the first large-scale nonhuman primate study of prion infectious titers in peripheral tissues from CJD patients,[56] studies using Met129 human PrP-expressing mice and sensitive detection assays have identified more infectivity than previously demonstrated.[123] As such, iatrogenic risk from non-CNS surgeries remains a theoretical possibility that cannot be ignored.

Patients with iatrogenic disease, when sCJD is the source, present with symptoms that are indistinguishable from sCJD, although patients exposed to contaminated dura mater have been reported to have kuru-like plaques on neuropathology.[202] *PRNP* variant 129MM is overrepresented in iatrogenic cases, analogous to sCJD cases, and heterozygous patients usually have longer incubation periods.[55]

Human-to-human transmission has also occurred orally and through blood transfusion. Oral infection occurred through the cannibalistic rituals of the Fore people in Papua New Guinea, where kuru was spread (see Evolution of the Prion Concept section). Three cases of clinical vCJD transmission from blood transfusion have been reported, although none of sporadic or genetic CJD[111,388] (https://www.cjd.ed.ac.uk/projects/transfusion-medicine-epidemiology-review-tmer). Of note, one patient who received a blood transfusion from a vCJD-infected donor died from a nonneurological disorder 5 years after transfusion but had PrP^d detectable in the spleen (not the brain). Whether this patient would have gone on to develop clinical vCJD is unknown but is probable. The three patients with clinical vCJD developed symptoms on average 7.5 ± 1 years after red cell transfusion. Interestingly, the donors were asymptomatic at the time of blood donation but developed vCJD symptoms 21-41 months after donation. There have been no reports of vertical transmission in CJD pregnancies, despite evidence for low-level PrP^d detection in gestational tissues.[120,218,241,372]

Zoonotic Transmission: Bovine Spongiform Encephalopathy and Variant Creutzfeldt-Jakob Disease

The only known zoonotic transmission of prion disease to humans has been from BSE, which gave rise to vCJD.[411] Classical BSE (C-BSE) was first identified as a prion disease in the mid-1980s in the United Kingdom,[406] and it is estimated that over 1 million infected cattle entered the human food system (https://www.who.int/csr/resources/publications/bse/en/BSEthreat.pdf). Prior to being discovered in humans, a novel prion disease was discovered in cats (feline spongiform encephalopathy; FSE), and C-BSE was shown to be transmissible to marmosets.[6,24] Then, in 1995, the first cases of vCJD were identified in humans.[36,52,411] Clinical features differed from sporadic CJD in that patients were younger, had longer disease duration, and more often presented with behavioral changes or painful paresthesias.[411] Brain isolates from C-BSE, FSE, and vCJD all produced the same clinical and neuropathological profile when transmitted to mice, proving the zoonotic link.[59] Cases of vCJD peaked in 2001-2002, with a total of 231 definite or probable cases worldwide to date.[177] While there have been no new cases since 2016, presumably due to a decline in BSE and restrictions on use of certain bovine tissues for human consumption, it is important to learn from the history of vCJD, so that we can be best prepared for any new zoonoses that may arise.

Once again, the *PRNP* 129 codon influenced the risk of vCJD, with almost all cases being MM, save for one MV case presenting in 2008[193] and one other MV patient who did not go on to autopsy confirmation. While these MV cases raised concern for a second wave of heterozygous patients with longer incubation periods, to date that has not occurred.

There is also the real potential of asymptomatic carriers. Given that vCJD is largely acquired orally, there is accumulation of PrP^d in extraneural tissues, such as the gut and lymphoid system. PrP^d is detectable in tonsils of vCJD patients, allowing diagnosis to be confirmed premortem by tonsil biopsy.[174] To survey the burden of PrP^d among people who would have been exposed to C-BSE during the epidemic, two studies were conducted in which researchers examined 45115 removed appendices or tonsils, looking for PrP^d by immunohistochemistry. Nineteen samples were positive,[153,175] giving a 1 in 2000 estimate of PrP^d carriers in the United Kingdom. Whether this really reflects infection or is a nonpathological accumulation of PrP is not clear. One difference between symptomatic vCJD cases and the asymptomatic cases associated with PrP^d deposition is that so-called carriers were represented by all codon 129 genotypes. A third appendix study examined 29516 tissues collected before the C-BSE epidemic (1962-1979) and from people born after 1996 who had appendices removed between 2000 and 2014. This study also revealed PrP^d deposits in 7 cases, raising two possibilities: (1) C-BSE exposure has occurred over a longer than expected timeframe or (2) abnormal PrP may accumulate in lymphoid tissues at a low background rate independent of BSE exposure.[154] The epidemiological consequence of these PrP^d deposition findings, with respect to clinical cases of vCJD and iatrogenic surgical risk, remains uncertain.

Since the C-BSE epidemic, other apparently sporadic but transmissible forms of BSE with different strain characteristics (eg, H-type BSE [H-BSE] and L-type BSE [L-BSE] or bovine amyloidotic spongiform encephalopathy [BASE]) have been identified.[30,68] Multiple studies of the transmissibility of L-BSE, H-BSE, and C-BSE in transgenic mice expressing human PrP^C have suggested that their relative zoonotic potentials are L-BSE > C-BSE > H-BSE.[215] However, no transmissions of atypical BSE strains to humans have been documented.

Potential Future Prion Zoonoses

No other zoonotic transmissions of prions have been reported to date, but CWD of cervids, including deer and elk, is increasing in prevalence, particularly in Canada and the United States (see Animal Prion Diseases section). However, there is ongoing human exposure, largely through consumption, raising concern that CWD could cross the species barrier and cause a new prion disease in humans. *In vitro* studies using protein misfolding cyclic amplification (PMCA) or RT-QuIC have demonstrated the production of human PrP aggregates after seeding with CWD prions.[31,114] *In vivo* experiments have been less convincing, with no transmission to mice expressing human PrP[206,319,338] and no transmission to macaques.[321] There was transmission to squirrel monkeys,[259,318] but these animals are less related to humans when compared with macaques. An important caveat with these studies is that there are several different CWD strains, and it is possible that some strains will be better able to cross the species barrier than others. New strains may also be generated through interspecies transmission to other mammals.[34,196,258] At the time of writing, the authors are aware of human PrP mouse and macaque *in vivo* experiments where preliminary data have suggested possible transmission, so the implications of CWD on human health remain unresolved. Finally, the recent discovery of prion disease in camels in Algeria has raised concerns of zoonotic risk from that species as well.[23]

Human Prion Disease: Diagnosis

In humans, prion disease diagnosis is based on a combination of clinical presentation, supporting diagnostic tests, and the absence of other explanations for a rapidly progressive dementia. Importantly, many tests and investigations are normal. Although prion diseases are infectious by definition, they do not induce the typical adaptive (humoral or cell-mediated) immune responses seen in infections caused by viruses, bacteria, fungi, parasites, etc., so there are usually no signs of inflammation on routine blood tests or cerebrospinal fluid (CSF) examination. This lack of response is due in large part to the fact that the disease is caused by an altered conformation of an endogenous protein, PrP, so it does not activate this type of immune response. There is, however, a considerable innate immune response in the form of astrocytosis and microglial activation in the brain,[244] which can be seen on pathology. For a definitive diagnosis, pathological confirmation is required, but brain biopsies are rarely done and can be falsely negative or nondiagnostic. Fortunately, there are specific tests that can help support a probable or possible diagnosis antemortem (Table 22.5).

TABLE 22.5 Human prion disease diagnostic criteria

Diagnostic Category	Criteria
Definite	Pathological confirmation: brain sample showing neuronal loss, spongiform change, astrocytosis, deposition of protease-resistant PrP; the presence of PrP^{Sc} may also be confirmed by immunoblot, where digestion and glycoform profiling help determine strain type
Probable	1. Neuropsychiatric disorder plus positive RT-QuIC (CSF or other tissue) **OR** 2. Rapidly progressive dementia; plus at least two of: • Myoclonus • Visual or cerebellar signs • Pyramidal/extrapyramidal signs • Akinetic mutism **AND** Positive result on at least one of the following: • EEG (electroencephalogram) periodic sharp wave complexes • 14-3-3 CSF assay in patients with a disease duration of <2 years • High signal in caudate/putamen on magnetic resonance imaging (MRI) brain scan or at least two cortical regions (temporal, parietal, occipital) either on diffusion-weighted imaging (DWI) or fluid-attenuated inversion recovery (FLAIR)
Possible	Progressive dementia **AND** at least two of the following: • Myoclonus • Visual or cerebellar signs • Pyramidal/extrapyramidal signs • Akinetic mutism **AND** duration of illness <2 years

Adapted from 2018 CDC guidelines. For both probable and possible disease, routine investigations must also exclude any alternate diagnosis.

Pathology

A diagnosis of definite prion disease requires tissue confirmation of protease-resistant PrP. For most forms of human prion disease, this requires postmortem brain autopsy. Antemortem brain biopsy can also confirm the diagnosis but is rarely used in suspect cases because of the challenges of decontaminating surgical instruments, and false negative or nondiagnostic sampling can occur. The classic neuropathological hallmarks of prion disease are neuronal loss, astrocytosis, spongiform change, and PrP^{d} deposition. Regional differences in pathology and PrP^{d} deposition patterns are seen depending on prion disease type (Fig. 22.12).

An exception to antemortem biopsy is vCJD, where tonsillar biopsy can provide a definite diagnosis. While the peak incidence of this disease has long passed, and the vast majority of human prion cases that clinicians will encounter will be sporadic, genetic, or other iatrogenic forms, it is theoretically possible that tonsillar biopsy could help diagnose future orally acquired prion zoonoses. For example, the cervid prion disease CWD (see section on Animal Prion Diseases), if able to cross the species barrier into humans, is likely to invade extra-CNS tissues, making tonsillar biopsies useful again.

Other tissues that have been used premortem for CJD diagnosis include skin biopsy[250,297] and nasal brushings,[292] but in these cases, it is not tissue pathology that is assessed, but rather the ability for prions in the tissue to trigger amplification of PrP misfolding in the RT-QuIC assay.[22,410] These approaches have yet to become mainstream but are being studied in several clinical research centers. At present, CSF is the preferred sample to collect for RT-QuIC, in part because evaluation of CSF is usually indicated in the work-up of other causes of rapidly progressive dementia, and it can be analyzed for other markers of inflammation, infection, and neoplasm.

Imaging

Neuroimaging is critical in the investigation of any rapidly progressive dementia, and the most useful imaging for prion disease diagnosis is magnetic resonance imaging (MRI). Of note, it is diffusion-weighted imaging (DWI) that is most diagnostic, but this sequence is not always included in routine MRI scans. On DWI MRI, the presence of restricted diffusion in at least two cerebral cortical regions and/or both the putamen and caudate has 83% sensitivity and specificity for CJD.[397,424] These diffusion changes at least partially correlate with the degree of spongiform change and PrP^{d} burden in these brain areas.[147] Recent protocols that quantify diffusion changes in 12 brain regions have led to improved accuracy and may allow for discrimination of subtypes of CJD.[46,304] Unfortunately, detecting this pattern depends on the inclusion of DWI sequences during acquisition, and the findings can be subtle and not always recognized by nonspecialist radiologists.[67] As such, an MRI reported as normal in a patient with CJD-like symptoms should be scrutinized by someone with experience reading CJD MRIs. In addition, MRI is less often abnormal in the genetic syndromes of FFI[86] and GSS.[379] Cortical ribboning restricted diffusion can also occur during status epilepticus, often in a strictly unilateral pattern. False-positive MRI findings have also been observed in treatable inflammatory conditions, Alzheimer disease, and dementia with Lewy bodies.[84,148,424]

Ligand-specific positron emission tomography (PET) for the prion protein is not available, but FDG-PET is being used more to investigate causes of neurodegeneration. For the rare cases of FFI, FDG-PET or SPECT may show thalamic hypometabolism that spreads to other areas with disease progression,[298] but there is no pathognomonic finding for CJD. Hypermetabolism can be seen in the limbic system, and parietal hypometabolism is a common finding, the latter associated with neuropathological findings of neuronal loss, astrocytosis, and spongiosis.[272] Because parietal lobe hypometabolism can be seen in other more common syndromes, including Alzheimer disease,[91] this particular finding is not helpful for diagnosis *per se*, but a longitudinal 2-year study of seven asymptomatic carriers of the *PRNP* G114V mutation and six related

noncarriers demonstrated progressive parietal and temporal lobe hypometabolism in asymptomatic carriers.[238]

Interestingly, these same patients were also imaged with diffusion tensor imaging (DTI). Over 2 years, the asymptomatic carriers also developed increased mean diffusivity along several white matter tracts, including several areas seen affected in symptomatic CJD patients. These included the superior longitudinal fasciculi, anterior thalamic radiations, cingulate gyri, and the left uncinate fasciculus.[190]

Electroencephalography

Electroencephalography (EEG) can be helpful if it shows 1-2 Hz periodic generalized discharges, as this has very high specificity (91%) for CJD in the right clinical context.[367] However, the sensitivity is much lower, around 44%, in part because not all types of CJD are associated with this finding,[109,173,424] and the finding itself is often transient, appearing later in disease, and disappearing within weeks to be replaced by background slowing. As such, the absence of discharges does not rule out CJD.

Cerebrospinal Fluid Analysis

A lumbar puncture is usually performed on any patient presenting with a rapidly progressive dementia in order to look for treatable infectious, inflammatory, neoplastic, or paraneoplastic causes that can mimic the CJD phenotype.[146] In cases of CJD, the CSF is usually normal or may have a slightly elevated protein level. The presence of white blood cells should raise the possibility of nonprion causes. In the past 10 years, a highly sensitive and specific test for prion disease has been used to assist in prion disease diagnosis: the RT-QuIC assay.[22,410] This assay detects trace amounts of misfolded pathogenic prion protein in the CSF by measuring its ability to seed the aggregation of normal recombinant prion protein substrate under shaking conditions. The assay is performed in the presence of thioflavin T (ThT), which fluoresces in the presence of β-sheet aggregates. A positive result is defined by a significant increase in ThT fluorescence. CSF RT-QuIC has high sensitivity and specificity (90%-97% and 99%, respectively)[48,137,138,173,293] for the most common forms of CJD and is being incorporated into more diagnostic criteria worldwide (Table 22.5). However, the RT-QuIC tests can be falsely negative if the spinal tap is contaminated with red blood cells. It has also been reported to be less sensitive for certain rare subtypes of sCJD (VV1 and MM2), sFI, and some genetic forms of prion disease.[48,173,339]

A previously used biomarker of prion disease was elevated 14-3-3 protein in the CSF. However, the sensitivity and specificity of this assay for CJD varies depending on assay type (Western blot vs ELISA) and subtype of CJD. Current ELISA assays that detect the gamma isoform have the highest sensitivities of 88%-97%,[224,344] but it is important to remember that the protein itself is more a marker of acute neuronal injury so if it is applied to control groups with inflammatory or neoplastic CNS diseases, specificity may be only 82%-87%.[219,368] This is an important point for clinicians who must be thorough in looking for treatable causes of rapid dementias.

In a population of 1000 subacute encephalopathy patients, CSF total tau levels >976 pg/mL, as measured by ELISA, was superior to 14-3-3, giving a sensitivity and specificity for CJD of 91% and 88%, respectively.[108] However, elevated total tau in the CSF can be detected in a number of neurodegenerative diseases as well as inflammatory and neoplastic CNS diseases, and there are no agreed upon cutoff values; therefore, total tau elevation must be interpreted in context. Interestingly, the ratio of phospho-tau to total tau may have better diagnostic value for CJD.[234,330,359]

Neurofilament light chain is being studied as a biomarker in many neurodegenerative diseases.[20] It is released in response to axonal damage and is not specific to any one disease. Its specificity for CJD requires further study,[173] but it appears to have higher sensitivity than 14-3-3 and total tau in detecting MV2 and VV2 subtypes.[2] It may also have prognostic value for genetic CJD patients, as it can be detected within 2 years of symptom onset.[382]

Human Prion Disease: Therapeutics

At present, there are no disease-modifying or preventative treatments available for prion diseases in humans, livestock, or wildlife. For patients with prion disease, clinicians can only offer symptom management. Decades of research have been invested in different therapeutic approaches,[378,391] from small molecule chemotherapeutic strategies targeting prion conversion,[357] to immunotherapy strategies[242] and newer approaches to neuroprotection based on stem cells.[327] These efforts started with demonstrations that certain polyanionic compounds could protect against scrapie infections in animals.[131,198] Extensive further screens have identified many classes of compounds that inhibit PrP^{Sc} formation in infected cells.[77,83,203,357,378] Many have been shown to increase survival time if given early to animals challenged with peripheral inoculations of prions. Unfortunately, most such compounds have poor bioavailability to the CNS unless they are injected directly into the brain. Inhibitors with some bioavailability to the brain, or that have been delivered directly into CJD patients' brains, have not yielded clear therapeutic effects.[100,391] Indeed, doing human trials on rare diseases that are phenotypically heterogeneous and rapidly progressive presents its own challenges. A major impediment to treating sporadic prion diseases (by far the most common) is that much brain damage is done before the patient shows clinical signs and is properly diagnosed. One promising development is the demonstration of antisense oligonucleotides as an approach for suppressing PrP^{C} and markedly slowing disease progression in prion-infected animals even when treatment is initiated near the onset of clinical signs.[277,284,326] This treatment, coupled with an international database of patients at risk for developing genetic CJD (prionregistry.org), and the possibility that plasma levels of neurofilament light chain levels may increase within 2 years of symptoms onset,[382] may set the stage for new human trials. Such trials would target patients with rare genetic forms of prion disease. A treatment that will rescue patients who are already symptomatic may be much further down the road and require combination therapy that targets prion formation and clearance, as well as neuroprotection.

Animal Prion Diseases

Bovine Spongiform Encephalopathy

The origin of the C-BSE is uncertain, but the ensuing epidemic was amplified to nearly 200 000 diagnosed cases by recycling of bovine meat and bone meal into cattle feed.[105] The vast majority of cases were in the United Kingdom, but some were seen in dozens of other counties.[177]

Fortunately, ruminant feed bans and other measures have now virtually eliminated C-BSE. However, H- and L-BSE cases continue to arise sporadically and, although rare and most prevalent in older cattle (>8 years old), they raise the possibility that without mitigation, further agriculturally mediated outbreaks of BSE or transmissions to other species might occur.

The feed-mediated amplification of C-BSE implies that most cases were due to oral exposures. Oral dosing experiments indicated that transmission of C-BSE to cattle can require as little as 1 mg of infected brain tissue.[207] After oral exposure, infectivity can be detected early in the ileal Peyer patches and then progress primarily via the autonomic nervous system to the brain prior to the onset of symptomatic disease.[3,177] In later stages, the infection disseminates further to other tissues including muscles.[3,26,107] Accordingly, some countries have designated multiple tissues as specified risk materials (SRMs) that should be excluded from human food, pharmaceuticals, and animal feeds to reduce the risk of transmission.

The atypical forms of BSE were discovered during surveillance of healthy and fallen stock. The designations of H- and L-BSE (or BASE) are based on the relative sizes (higher vs lower) of protease-treated PrP^{Res} bands on immunoblots.[105,107,177] Although the causes of these atypical types of BSE remain uncertain, the rarity and age-dependence of their incidence are consistent with spontaneous, rather than acquired, etiologies. Both L- and H-BSE are transmissible as distinct prion strains via intracerebral inoculation,[30,62,208,236] but transmission of H-BSE to mice expressing bovine PrP has sometimes produced a C-BSE-like strain.[384] Oral transmission of L-BSE to cattle has been inefficient, with a low clinical attack rate.[290] L- and H-BSE PrP^{d} are found most abundantly in the central and peripheral nervous systems with little accumulation in lymphoid organs (reviewed in Refs.[107,177,215]).

Scrapie in Small Ruminants

As with BSE, scrapie strains in sheep have been described as “classical” or “atypical” with the latter referring to the Nor98 strain.[38,70,177,257] The occurrence of scrapie in small ruminants extends to many parts of the world, including 25 countries in the EU, with a prevalence of 9 and 6 cases per 10 000 tested animals for typical and atypical scrapie, respectively.[177] Interestingly, atypical scrapie also has been detected in Australia and New Zealand, countries that have been regarded as free of classical scrapie. The prevalence of classical scrapie varies within countries and over time, consistent with contagion between infected animals being the main etiology. In contrast, the prevalence of atypical scrapie is more consistent, regardless of exposure. Such findings support the notion that although atypical scrapie is experimentally transmissible, it is not as naturally contagious as classical scrapie and may be largely spontaneous.

Differences in the tissue distributions of classical and atypical scrapie PrP^{Sc} may help to explain the apparent differences in the natural contagiousness of these strains.[70,177] In classical scrapie, the infection is usually contracted orally within the first weeks of life. The infection enters gut-associated lymphoid tissue such as the Peyer patches and then spreads to other lymphoid organs, and on to the spinal cord and brain via the autonomic nervous system. Eventually, the infection spreads centrifugally to other tissues such as the peripheral nervous system, muscle, and blood. Pregnant ewes can accumulate infectivity in their placentas, which is shed into the environment as the afterbirth. Infectivity is also found in colostrum and milk. As a result, perinatal exposures contribute significantly to the natural spread of classical scrapie within flocks.

The distribution of atypical scrapie prions is more restricted to the central nervous system, presumably limiting the potential for environment shedding or transmission via casual contact,[177] but atypical scrapie can be transmitted experimentally via the oral route, raising the possibility that this might occur naturally to some extent.

Sheep have polymorphisms at *PRNP* codons 136, 154, and 171 that can influence susceptibility to scrapie (Fig. 22.13), with those homozygous for the A136, R154, R171 (ARR) allele being particularly resistant to scrapie.[70,177] Indeed, the resistance of this sheep genotype to classical scrapie has been used as justification for breeding programs aimed at generating scrapie-resistant flocks. Such programs have markedly reduced the prevalence of scrapie in Europe[15] and the United States.[12] However, a note of caution is that carriers of the ARR allele are susceptible to atypical Nor98 scrapie.[38] Also of concern is the emergence of a C-BSE–like prion strain in asymptomatic pigs inoculated with atypical (Nor98) sheep scrapie.[258] Such findings emphasize that resistance of host genotypes to prions can be strain dependent and that transmissions into intermediate species can alter prion strain characteristics and, perhaps, zoonotic potential.

Chronic Wasting Disease

Chronic wasting disease (CWD) is a prion disease of cervids that was first identified in 1967 in a captive mule deer in Colorado.[415] Since that time, it has steadily spread, both through transport of infected captive deer and elk and through wild animal migration, to at least 26 U.S. states, 3 Canadian provinces, and has affected cervids in Finland, Norway, Sweden, and South Korea (https://www.cdc.gov/prions/cwd/occurrence.html). The cervids affected include deer, elk, moose, and reindeer. CWD is the most efficiently transmitted prion disease and is now the most prevalent prion disease, with cases continuing to rise. Up to 30% of animals are infected in some free-ranging herds, and rates approach 100% in some captive deer herds. Management has proven difficult because CWD is being spread throughout the extensive geographic ranges of wild deer and elk, and CWD prions cannot be easily cleared from the environment, where they avidly bind soil and likely remain infectious for years.[216]

As with typical scrapie, CWD transmission is largely by the oral route. Prions can be found in many tissues of CWD-affected cervids, including the central nervous system, lymphoid tissues, spleen and pancreas, peripheral nerves, muscle, blood, and antler velvet.[129] Prions are shed into the environment through saliva, feces, urine, placenta, and carcasses, leading to environmental contamination and horizontal transmission.[260] There is also evidence for vertical transmission.[282,347] The fact that CWD prions are present in muscle[10] and elk velvet,[11] raise particular concern for human exposure and potential zoonotic transmission.

Several polymorphisms in the cervid *PRNP* gene have been linked to reduced CWD susceptibility, delayed disease onset, altered disease course, and/or pathology (Fig. 22.13).[13,14,278,285] These include alleles encoding leucine (L) at residue 132 in North American elk, asparagine (N) at residue 138 in reindeer or caribou, phenylalanine (F) at residue 225 in mule deer, and serine (S) at residue 96 in whitetail deer. Early results from a breeding strategy to reduce the frequency of the highly susceptible 96G (glycine at residue 96) *PRNP* variant in farmed whitetail deer have been promising with respect to CWD prevalence.[165]

Camel Prion Disease

Recently, prion disease has been discovered in three dromedary camels (>8 years of age) brought to a slaughterhouse in Algeria.[23] These animals had pathognomonic neurodegeneration and PrP^d in brain tissue. The origin of camel prion disease is unknown, but detection of PrP^d in lymphoid tissue increases the chances of horizontal spreading among individuals. A retrospective analysis indicated that 3.1% of camels coming to the slaughterhouse in 2015-2016 had neurological signs suggesting prion disease.

Other Mammalian Prion Diseases

Prion diseases of humans, cattle, sheep, and cervids have been transmitted experimentally or inadvertently to a wide range of additional mammalian species including nonhuman primates, mink (transmissible mink encephalopathy), cats (feline spongiform encephalopathy), pigs, rodents, and exotic ungulates.[4,353] Of note, some species are susceptible to an unusually broad range of prion strains, that is, bank voles[287,403] and squirrel monkeys,[56,259,321] while others are largely resistant, for example, dogs.[281,396] However, it is not clear whether these additional species naturally harbor their own prion disorders. Gianluigi Zanusso and colleagues revealed an intriguing situation in which a man and his cat had simultaneous prion diseases, with the man's disease like sCJD and the cat's being distinct from the feline spongiform encephalopathy resulting from BSE infections.[423] However, the origin(s) of these two cases remains unknown.

Given the diversity of prion strains in mammals, practical concerns include the extent to which interspecies exposures to prion-infected materials might generate prion strains with new transmission and virulence characteristics. Examples of such shifts in prion strain characteristics upon interspecies transmission are well documented.[34,196,258] A related concern is whether CWD is transmissible from cervids into predators, scavengers, other wildlife, or domesticated species. This appears not to be the case for mountain lions at least.[418] However, mountain lions, crows, and coyotes can shed prions that they have consumed in their feces for a few days, potentially contributing to environmental dissemination.[37] On a positive note, the prion seeding activity shed from mountain lions is only ~3% of that consumed, indicating an ability to either degrade or sequester ingested CWD prions.

Detection of Prions

The detection of prions, or surrogates thereof, is important in many facets of prion disease management, including surveillance, risk reduction, diagnosis, therapeutics, and fundamental research. Historically, prions have been detected by animal bioassays taking months to years. Much more rapid immunoassays, such as ELISA, Western blot, or immunohistochemistry, can now be used for prion (or PrP^d) detection in specimens with high prion loads, such as nervous or lymphoid tissues.[265] However, up to 10 million-fold higher sensitivities, even exceeding those of animal bioassays, can be obtained with cell-free prion seed amplification assays such as PMCA,[334] amyloid seeding assay,[99] or RT-QuIC.[22,135,265,410] Certain murine prions can also be assayed with high sensitivity using scrapie cell assays[201] that involve prion amplification through multiple *in vitro* passages followed by *in situ* immune detection of infected cells.

Many adaptations of cell-free amplification assays have been developed for most mammalian prion strains.[135,294] Although amplification assays are more time-consuming and technically demanding than ELISA assays, they provide the sensitivity necessary for detecting low levels of prion seeds in diagnostic specimens that can be collected from live individuals or released into the environment. As noted above, the most commonly used amplification assay platform for human diagnostics is RT-QuIC. *Antemortem* diagnostics for animal prion diseases are under development[70,134,166,167] and, if sufficiently practical and validated, may become particularly important in the determination of prion infection status in cervids and sheep as part of surveillance and prion-free certification programs. One approach to testing for CWD in live cervids has been to analyze rectoanal mucosal lymphoid–associated tissue (RAMALT) biopsied from anesthetized animals.[167] Several studies have demonstrated markedly improved diagnostic sensitivity when RAMALT biopsies are tested with RT-QuIC vs immunohistochemistry.[166] Another promising but as yet unvalidated approach is RT-QuIC testing of outer ear punches.[134]

Antemortem diagnosis has not only been difficult historically for PrP-based prion diseases but also for many of the other human proteinopathies. Accordingly, RT-QuIC–like seed amplification assays are being developed for many of these other diseases as well.[135]

Inactivation of Prions

Prions are often more resistant to inactivation than typical microbes and may resist decontamination procedures that are routine in clinical, agricultural, or mortuary settings. However, certain chemical treatments, prolonged high temperature autoclaving, and thorough incineration, alone or in combination, can be effective in decontaminating contaminated materials and surfaces. The reader is referred to the Biosafety in Microbiological and Biomedical Laboratories 6th edition for details and recommended handling protocols.[76]

Perspectives

Major challenges remain to be tackled in the prion diseases field. We currently have three near-atomic structures of two rodent prion strains, but there are dozens more to be determined to fully understand prion diversity. Research into the pathogenesis of prions has been rich and multifaceted. However, it remains unclear how to prioritize the many ways that prions can be neurotoxic, and how to translate such information into practical therapeutics. Although prion disease diagnostics in living human patients have improved markedly in recent years, much remains to be done to make *antemortem* diagnostics in other mammals accurate and practical enough for routine applications in agriculture and wildlife. Further study is also required to understand the extent to which prionlike mechanisms contribute to the etiology and pathogenesis of the many proteinopathies that involve the abnormal assembly of proteins other than PrP.

In assessing the clinical significance of prionlike characteristics of various protein aggregates, a key issue is not only transmissibility but also pathogenicity; that is, do replicating and spreading aggregates necessarily cause disease in the new host[75,93]? This issue is analogous to the distinction between virulent vs nonvirulent viral or bacterial infections. When transmitting TSE prions from one species to another, or when inoculating synthetic PrP aggregates, the inocula can sometimes initiate extensive conversion of the host's endogenous PrP molecules without causing clinical disease or, even TSE-associated neuropathological lesions, within the lifespan of the host.[32,75,163,213,246,322] Indeed, different

wild-type hamster PrP amyloid fibrils have been shown to range in lethality across many orders of magnitude and have varying abilities to replicate and/or cause neuropathology (Fig. 22.15). Also, some neuropathogenic mutant forms of PrP can accumulate in the host without being transmissible to other hosts.[93]

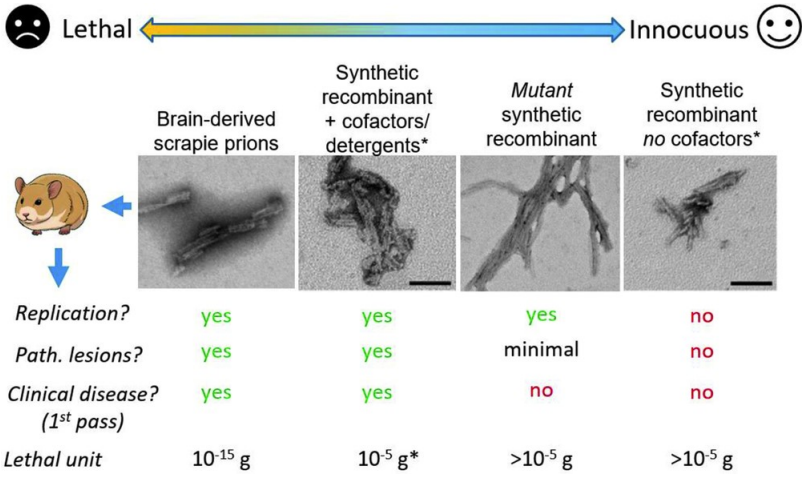

FIGURE 22.15 **Range of transmissibility and pathogenicity of hamster PrP amyloid fibrils**. Although all of the depicted amyloid fibrils are capable of continuous propagation either *in vivo* or *in vitro*, they differ dramatically in their infectious properties when inoculated intracerebrally into hamsters. Negatively stained transmission electron micrographs show fibrillar ultrastructures (bar = 100 nm). Although the fibrils depicted in the second panel from left were prepared with detergent and only barely lethal,[195] it is important to note that other synthetic recombinant prions prepared with cofactors can be orders of magnitude more lethal per unit protein (lethal unit of ~10^{-10} g).[400]

(Adapted from Figure 1 of Caughey B, Kraus A. Transmissibility versus pathogenicity of self-propagating protein aggregates. *Viruses*. 2019;11(11):1044. http://creativecommons.org/licenses/by/4.0/.)

In humans, as noted above, concerns remain about the implications of finding abnormal PrP deposits in the appendices of 1 out of every 2000 Britons in an age cohort that could have been exposed to BSE prions. Related concerns about the potential transmissibility of Aβ amyloid have been raised recently based on the detection of Aβ pathology in patients with early-onset CAA or who had undergone prior neurosurgeries,[184] dura mater grafts,[168] or injections of cadaveric growth hormone.[183] However, an extensive analysis of a cohort of people in the latter category provided evidence that exposures to injected Aβ or α-synuclein amyloids were not epidemiologically significant causes of neurological disease.[18,181] These examples emphasize the epidemiological importance of considering not only prionlike spreading potential but also the clinical manifestations, if any, of accumulating self-propagating protein assemblies.

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REFERENCES

1. Abdelaziz DH, Abdulrahman BA, Gilch S, Schatzl HM. Autophagy pathways in the treatment of prion diseases. Curr Opin Pharmacol. 2019;44:46-52.

2. Abu-Rumeileh S, Capellari S, Stanzani-Maserati M, et al. The CSF neurofilament light signature in rapidly progressive neurodegenerative dementias. Alzheimers Res Ther. 2018;10(1):3.

3. Ackermann I, Ulrich R, Tauscher K, et al. Prion infectivity and PrP(BSE) in the peripheral and central nervous system of cattle 8 months post oral BSE challenge. Int J Mol Sci. 2021;22(21):11310.

4. Aguilar-Calvo P, Garcia C, Espinosa JC, Andreoletti O, Torres JM. Prion and prion-like diseases in animals. Virus Res. 2015;207:82-93.

5. Aiken JM, Williamson JL, Marsh RF. Evidence of mitochondrial involvement in scrapie infection. J Virol. 1989;63:1686-1694.

6. Aldhous P. BSE: spongiform encephalopathy found in cat. Nature. 1990;345(6272):194.

7. Alper T, Cramp WA, Haig DA, Clarke MC. Does the agent of scrapie replicate without nucleic acid? Nature. 1967;214:764-766.

8. Amin L, Nguyen XT, Rolle IG, et al. Characterization of prion protein function by focal neurite stimulation. J Cell Sci. 2016;129(20):3878-3891.

9. Andreoletti O, Simon S, Lacroux C, et al. PrPSc accumulation in myocytes from sheep incubating natural scrapie. Nat Med. 2004;10(6):591-593.

10. Angers RC, Browning SR, Seward TS, et al. Prions in skeletal muscles of deer with chronic wasting disease. Science. 2006;311(5764):1117.

11. Angers RC, Seward TS, Napier D, et al. Chronic wasting disease prions in elk antler velvet. Emerg Infect Dis. 2009;15(5):696-703.

12. APHIS USDoA. National Scrapie Eradication Program August 2021 Monthly Report. 2021. https://www.aphis.usda.gov/animal_health/animal_diseases/scrapie/downloads/monthly_scrapie_report.pdf

13. Arifin MI, Hannaoui S, Chang SC, Thapa S, Schatzl HM, Gilch S. Cervid prion protein polymorphisms: role in chronic wasting disease pathogenesis. Int J Mol Sci. 2021;22(5):2271.

14. Arifin MI, Staskevicius A, Shim SY, et al. Large-scale prion protein genotyping in Canadian caribou populations and potential impact on chronic wasting disease susceptibility. Mol Ecol. 2020;29(20):3830-3840.

15. Arnold M, Ortiz-Pelaez A. The evolution of the prevalence of classical scrapie in sheep in Great Britain using surveillance data between 2005 and 2012. Prev Vet Med. 2014;117(1):242-250.

16. Asante EA, Gowland I, Grimshaw A, et al. Absence of spontaneous disease and comparative prion susceptibility of transgenic mice expressing mutant human prion proteins. J Gen Virol. 2009;90(Pt 3):546-558.

17. Asante EA, Smidak M, Grimshaw A, et al. A naturally occurring variant of the human prion protein completely prevents prion disease. Nature. 2015;522(7557):478-481.

18. Asher DM, Belay E, Bigio E, et al. Risk of transmissibility from neurodegenerative disease-associated proteins: experimental knowns and unknowns. J Neuropathol Exp Neurol. 2020;79(11):1141-1146.

19. Ashok A, Hegde RS. Selective processing and metabolism of disease-causing mutant prion proteins. PLoS Pathog. 2009;5(6):e1000479.

20. Ashton NJ, Hye A, Rajkumar AP, et al. An update on blood-based biomarkers for non-Alzheimer neurodegenerative disorders. Nat Rev Neurol. 2020;16(5):265-284.

21. Atarashi R, Moore RA, Sim VL, et al. Ultrasensitive detection of scrapie prion protein using seeded conversion of recombinant prion protein. Nat Methods. 2007;4(8):645-650.

22. Atarashi R, Satoh K, Sano K, et al. Ultrasensitive human prion detection in cerebrospinal fluid by real-time quaking-induced conversion. Nat Med. 2011;17(2):175-178.

23. Babelhadj B, Di Bari MA, Pirisinu L, et al. Prion disease in dromedary camels, Algeria. Emerg Infect Dis. 2018;24(6):1029-1036.

24. Baker HF, Ridley RM, Wells GAH. Experimental transmission of BSE and scrapie to the common marmoset. Vet Rec. 1993;132:403-406.

25. Baldauf E, Beekes M, Diringer H. Evidence for an alternative direct route of access for the scrapie agent to the brain bypassing the spinal cord. J Gen Virol. 1997;78 (Pt 5):1187-1197.

26. Balkema-Buschmann A, Fast C, Kaatz M, et al. Pathogenesis of classical and atypical BSE in cattle. Prev Vet Med. 2011;102(2):112-117.

27. Banks WA, Niehoff ML, Adessi C, Soto C. Passage of murine scrapie prion protein across the mouse vascular blood-brain barrier. Biochem Biophys Res Commun. 2004;318(1):125-130.

28. Baron GS, Hughson AG, Raymond GJ, et al. Effect of glycans and the glycophosphatidylinositol anchor on strain dependent conformations of scrapie prion protein: improved purifications and infrared spectra. Biochemistry. 2011;50(21):4479-4490.

29. Baron GS, Wehrly K, Dorward DW, Chesebro B, Caughey B. Conversion of raft associated prion protein to the protease-resistant state requires insertion of PrP-res (PrP(Sc)) into contiguous membranes. EMBO J. 2002;21(5):1031-1040.

30. Baron TG, Biacabe AG, Bencsik A, Langeveld JP. Transmission of new bovine prion to mice. Emerg Infect Dis. 2006;12(7):1125-1128.

31. Barria MA, Libori A, Mitchell G, Head MW. Susceptibility of human prion protein to conversion by chronic wasting disease prions. Emerg Infect Dis. 2018;24(8):1482-1489.

32. Barron RM, King D, Jeffrey M, et al. PrP aggregation can be seeded by pre-formed recombinant PrP amyloid fibrils without the replication of infectious prions. Acta Neuropathol. 2016;132(4):611-624.

33. Bartz JC. Prion strain diversity. Cold Spring Harb Perspect Med. 2016;6(12):a024349.

34. Bartz JC, Bessen RA, McKenzie D, Marsh RF, Aiken JM. Adaptation and selection of prion protein strain conformations following interspecies transmission of transmissible mink encephalopathy. J Virol. 2000;74(12):5542-5547.

35. Baskakov IV, Caughey B, Requena JR, Sevillano AM, Surewicz WK, Wille H. The prion 2018 round tables (I): the structure of PrP(Sc). Prion. 2019;13(1):46-52.

36. Bateman D, Hilton D, Love S, Zeidler M, Beck J, Collinge J. Sporadic Creutzfeldt-Jakob disease in a 18-year-old in the UK. Lancet. 1995;346(8983):1155-1156.

37. Baune C, Wolfe LL, Schott KC, et al. Reduction of chronic wasting disease prion seeding activity following digestion by mountain lions. mSphere. 2021;6(6):e0081221.

38. Benestad SL, Arsac JN, Goldmann W, Noremark M. Atypical/Nor98 scrapie: properties of the agent, genetics, and epidemiology. Vet Res. 2008;39(4):19.

39. Benestad SL, Austbo L, Tranulis MA, Espenes A, Olsaker I. Healthy goats naturally devoid of prion protein. Vet Res. 2012;43:87.

40. Beraldo FH, Ostapchenko VG, Caetano FA, et al. Regulation of amyloid beta oligomer binding to neurons and neurotoxicity by the prion protein-mGluR5 complex. J Biol Chem. 2016;291(42):21945-21955.

41. Beraldo FH, Ostapchenko VG, Xu JZ, et al. Mechanisms of neuroprotection against ischemic insult by stress-inducible phosphoprotein-1/prion protein complex. J Neurochem. 2018;145(1):68-79.

42. Bernoulli C, Sigfried J, Baumgartner G, et al. Danger of accidental person to person transmission of Creutzfeldt-Jakob disease by surgery. Lancet. 1977;1:478-479.

43. Bessen RA, Kocisko DA, Raymond GJ, Nandan S, Lansbury PT Jr, Caughey B. Nongenetic propagation of strain-specific phenotypes of scrapie prion protein. Nature. 1995;375:698-700.

44. Bessen RA, Marsh RF. Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy. J Virol. 1994;68:7859-7868.

45. Biasini E, Turnbaugh JA, Unterberger U, Harris DA. Prion protein at the crossroads of physiology and disease. Trends Neurosci. 2012;35(2):92-103.

46. Bizzi A, Pascuzzo R, Blevins J, et al. Subtype diagnosis of sporadic Creutzfeldt-Jakob disease with diffusion magnetic resonance imaging. Ann Neurol. 2021;89(3):560-572.

47. Bolton DC, McKinley MP, Prusiner SB. Identification of a protein that purifies with the scrapie prion. Science. 1982;218:1309-1311.

48. Bongianni M, Orrù CD, Groveman BR, et al. Diagnosis of human prion disease using real-time quaking-induced conversion testing of olfactory mucosa and cerebrospinal fluid samples. JAMA Neurol. 2017;74(2):1-8.

49. Borchelt DR, Scott M, Taraboulos A, Stahl N, Prusiner SB. Scrapie and cellular prion proteins differ in the kinetics of synthesis and topology in cultured cells. J Cell Biol. 1990;110:743-752.

50. Borchelt DR, Taraboulos A, Prusiner SB. Evidence for synthesis of scrapie prion protein in the endocytic pathway. J Biol Chem. 1992;267:16188-16199.

51. Brandner S, Isenmann S, Raeber A, et al. Normal host prion protein necessary for scrapie-induced neurotoxicity. Nature. 1996;379:339-343.

52. Britton TC, al-Sarraj S, Shaw C, Campbell T, Collinge J. Sporadic Creutzfeldt-Jakob disease in a 16-year-old in the UK. Lancet. 1995;346(8983):1155.

53. Brown DR, Qin K, Herms JW, et al. The cellular prion protein binds copper in vivo. Nature. 1997;390:684-687.

54. Brown P, Bradley R. 1755 and all that: a historical primer of transmissible spongiform encephalopathy. BMJ. 1998;317(7174):1688-1692.

55. Brown P, Brandel JP, Sato T, et al. Iatrogenic Creutzfeldt-Jakob disease, final assessment. Emerg Infect Dis. 2012;18(6):901-907.

56. Brown P, Gibbs CJ Jr, Rodgers-Johnson P, et al. Human spongiform encephalopathy: the National Institutes of Health series of 300 cases of experimentally transmitted disease. Ann Neurol. 1994;35:513-529.

57. Brownell B, Oppenheimer DR. An ataxic form of subacute presenile polioencephalopathy (Creutzfeldt-Jakob disease). J Neurol Neurosurg Psychiatry. 1965;28:350-361.

58. Bruce M, Dickinson AG. Biological stability of different classes of scrapie agent. In: Prusiner SB, Hadlow WJ, eds. Slow Transmissible Diseases of the Nervous System. Academic Press; 1979:71-86.

59. Bruce ME, Will RG, Ironside JW, et al. Transmissions to mice indicate that ‘new variant’ CJD is caused by the BSE agent. Nature. 1997;389:498-501.

60. Bueler H, Aguzzi A, Sailer A, et al. Mice devoid of PrP are resistant to scrapie. Cell. 1993;73:1339-1347.

61. Bueler H, Fischer M, Lang Y, et al. Normal development and behavior of mice lacking the neuronal cell-surface PrP protein. Nature. 1992;356:577-582.

62. Buschmann A, Gretzschel A, Biacabe AG, et al. Atypical BSE in Germany—proof of transmissibility and biochemical characterization. Vet Microbiol. 2006;117(2-4):103-116.

63. Callender JA, Sevillano AM, Soldau K, et al. Prion protein post-translational modifications modulate heparan sulfate binding and limit aggregate size in prion disease. Neurobiol Dis. 2020;142:104955.

64. Cancellotti E, Bradford BM, Tuzi NL, et al. Glycosylation of PrPC determines timing of neuroinvasion and targeting in the brain following transmissible spongiform encephalopathy infection by a peripheral route. J Virol. 2010;84(7):3464-3475.

65. Carroll JA, Groveman BR, Williams K, Moore R, Race B, Haigh CL. Prion protein N1 cleavage peptides stimulate microglial interaction with surrounding cells. Sci Rep. 2020;10(1):6654.

66. Carroll JA, Race B, Williams K, Striebel J, Chesebro B. Microglia are critical in host defense against prion disease. J Virol. 2018;92(15):e00549-18.

67. Carswell C, Thompson A, Lukic A, et al. MRI findings are often missed in the diagnosis of Creutzfeldt-Jakob disease. BMC Neurol. 2012;12:153.

68. Casalone C, Zanusso G, Acutis P, et al. Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. Proc Natl Acad Sci U S A. 2004;101(9):3065-3070.

69. Cashman NR, Loertscher R, Nalbantoglu J, et al. Cellular isoform of the scrapie agent protein participates in lymphocyte activation. Cell. 1990;61:185-192.

70. Cassmann ED, Greenlee JJ. Pathogenesis, detection, and control of scrapie in sheep. Am J Vet Res. 2020;81(7):600-614.

71. Castilla J, Saa P, Hetz C, Soto C. In vitro generation of infectious scrapie prions. Cell. 2005;121(2):195-206.

72. Caughey B, Baron GS. Prions and their partners in crime. Nature. 2006;443(7113):803-810.

73. Caughey B, Baron GS, Chesebro B, Jeffrey M. Getting a grip on prions: oligomers, amyloids, anchors and pathological membrane interactions. Annu Rev Biochem. 2009;78:177-204.

74. Caughey B, Kocisko DA, Raymond GJ, Lansbury PT. Aggregates of scrapie associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistant state. Chem Biol. 1995;2:807-817.

75. Caughey B, Kraus A. Transmissibility versus pathogenicity of self-propagating protein aggregates. Viruses. 2019;11(11):1044.

76. Caughey B, Priola SA, Schonberger LB, Asher D. Prion diseases. In: Meechan PJ, Potts J, eds. Biosafety in Microbiological and Biomedical Laboratories. 6th ed. U.S. Department of Health and Human Services; 2020:355-366.

77. Caughey B, Race RE. Potent inhibition of scrapie-associated PrP accumulation by Congo red. J Neurochem. 1992;59:768-771.

78. Caughey B, Race RE, Ernst D, Buchmeier MJ, Chesebro B. Prion protein (PrP) biosynthesis in scrapie-infected and uninfected neuroblastoma cells. J Virol. 1989;63:175-181.

79. Caughey B, Raymond GJ. The scrapie-associated form of PrP is made from a cell surface precursor that is both protease- and phospholipase-sensitive. J Biol Chem. 1991;266:18217-18223.

80. Caughey B, Raymond GJ. Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells. J Virol. 1993;67:643-650.

81. Caughey B, Raymond GJ, Ernst D, Race RE. N-terminal truncation of the scrapie-associated form of PrP by lysosomal protease(s): implications regarding the site of conversion of PrP to the protease-resistant state. J Virol. 1991;65:6597-6603.

82. Caughey BW, Dong A, Bhat KS, Ernst D, Hayes SF, Caughey WS. Secondary structure analysis of the scrapie-associated protein PrP 27-30 in water by infrared spectroscopy. Biochemistry. 1991;30:7672-7680.

83. Caughey WS, Raymond LD, Horiuchi M, Caughey B. Inhibition of protease-resistant prion protein formation by porphyrins and phthalocyanines. Proc Natl Acad Sci U S A. 1998;95(21):12117-12122.

84. Chang CC, Eggers SD, Johnson JK, Haman A, Miller BL, Geschwind MD. Anti-GAD antibody cerebellar ataxia mimicking Creutzfeldt-Jakob disease. Clin Neurol Neurosurg. 2007;109(1):54-57.

85. Chassefeyre R, Chaiamarit T, Verhelle A, et al. Endosomal sorting drives the formation of mutant prion endoggresomes. Sci Adv. 2021;7(52):eabg3693.

86. Chen C, Wang JC, Shi Q, et al. Analyses of the survival time and the influencing factors of Chinese patients with prion diseases based on the surveillance data from 2008-2011. PLoS One. 2013;8(5):e62553.

87. Chesebro B, Race B, Meade-White K, et al. Fatal transmissible amyloid encephalopathy: a new type of prion disease associated with lack of prion protein membrane anchoring. PLoS Pathog. 2010;6(3):e1000800.

88. Chesebro B, Race R, Wehrly K, et al. Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain. Nature. 1985;315:331-333.

89. Chesebro B, Striebel J, Rangel A, et al. Early generation of new PrPSc on blood vessels after brain microinjection of scrapie in mice. MBio. 2015;6(5):e01419-15.

90. Chesebro B, Trifilo M, Race R, et al. Anchorless prion protein results in infectious amyloid disease without clinical scrapie. Science. 2005;308(5727):1435-1439.

91. Chetelat G, Arbizu J, Barthel H, et al. Amyloid-PET and (18)F-FDG-PET in the diagnostic investigation of Alzheimer's disease and other dementias. Lancet Neurol. 2020;19(11):951-962.

92. Chiesa R. The elusive role of the prion protein and the mechanism of toxicity in prion disease. PLoS Pathog. 2015;11(5):e1004745.

93. Chiesa R, Piccardo P, Quaglio E, et al. Molecular distinction between pathogenic and infectious properties of the prion protein. J Virol. 2003;77(13):7611-7622.

94. Chiti F, Dobson CM. Protein misfolding, functional amyloid, and human disease. Annu Rev Biochem. 2006;75:333-366.

95. Choi SI, Ju WK, Choi EK, et al. Mitochondrial dysfunction induced by oxidative stress in the brains of hamsters infected with the 263 K scrapie agent. Acta Neuropathol. 1998;96(3):279-286.

96. Cobb NJ, Apostol MI, Chen S, Smirnovas V, Surewicz WK. Conformational stability of mammalian prion protein amyloid fibrils is dictated by a packing polymorphism within the core region. J Biol Chem. 2014;289(5):2643-2650.

97. Cobb NJ, Sonnichsen FD, McHaourab H, Surewicz WK. Molecular architecture of human prion protein amyloid: a parallel, in-register beta-structure. Proc Natl Acad Sci U S A. 2007;104(48):18946-18951.

98. Colby DW, Wain R, Baskakov IV, et al. Protease-sensitive synthetic prions. PLoS Pathog. 2010;6(1):e1000736.

99. Colby DW, Zhang Q, Wang S, et al. Prion detection by an amyloid seeding assay. Proc Natl Acad Sci U S A. 2007;104(52):20914-20919.

100. Collinge J, Gorham M, Hudson F, et al. Safety and efficacy of quinacrine in human prion disease (PRION-1 study): a patient-preference trial. Lancet Neurol. 2009;8(4):334-344.

101. Collinge J, Palmer MS, Sidle KC, et al. Transmission of fatal familial insomnia to laboratory animals. Lancet. 1995;346(8974):569-570.

102. Collins S, Law MG, Fletcher A, Boyd A, Kaldor J, Masters CL. Surgical treatment and risk of sporadic Creutzfeldt-Jakob disease: a case-control study. Lancet. 1999;353(9154):693-697.

103. Collins SJ, Tumpach C, Groveman BR, Drew SC, Haigh CL. Prion protein cleavage fragments regulate adult neural stem cell quiescence through redox modulation of mitochondrial fission and SOD2 expression. Cell Mol Life Sci. 2018;75(17):3231-3249.

104. Corbett GT, Wang Z, Hong W, et al. PrP is a central player in toxicity mediated by soluble aggregates of neurodegeneration-causing proteins. Acta Neuropathol. 2020;139(3):503-526.

105. Corona C, Vallino Costassa E, Iulini B, et al. Phenotypical variability in bovine spongiform encephalopathy: epidemiology, pathogenesis, and diagnosis of classical and atypical forms. Prog Mol Biol Transl Sci. 2017;150:241-265.

106. Cortez LM, Nemani SK, Duque Velasquez C, et al. Asymmetric-flow field-flow fractionation of prions reveals a strain-specific continuum of quaternary structures with protease resistance developing at a hydrodynamic radius of 15 nm. PLoS Pathog. 2021;17(6):e1009703.

107. Costassa EV, Iulini B, Mazza M, et al. Pathogenesis and transmission of classical and atypical BSE in cattle. Food Saf (Tokyo). 2016;4(4):130-134.

108. Coulthart MB, Jansen GH, Olsen E, et al. Diagnostic accuracy of cerebrospinal fluid protein markers for sporadic Creutzfeldt-Jakob disease in Canada: a 6-year prospective study. BMC Neurol. 2011;11:133.

109. Cracco L, Appleby BS, Gambetti P. Fatal familial insomnia and sporadic fatal insomnia. Handb Clin Neurol. 2018;153:271-299.

110. Cracco L, Notari S, Cali I, et al. Novel strain properties distinguishing sporadic prion diseases sharing prion protein genotype and prion type. Sci Rep. 2017;7:38280.

111. Crowder LA, Schonberger LB, Dodd RY, Steele WR. Creutzfeldt-Jakob disease lookback study: 21 years of surveillance for transfusion transmission risk. Transfusion. 2017;57(8):1875-1878.

112. Cruz M, Mahillo-Fernandez I, Rabano A, et al. Late-in-life surgery associated with Creutzfeldt-Jakob disease: a methodological outline for evidence-based guidance. Emerg Themes Epidemiol. 2013;10(1):5.

113. Cunningham C, Deacon R, Wells H, et al. Synaptic changes characterize early behavioural signs in the ME7 model of murine prion disease. Eur J Neurosci. 2003;17(10):2147-2155.

114. Davenport KA, Henderson DM, Bian J, Telling GC, Mathiason CK, Hoover EA. Insights into chronic wasting disease and bovine spongiform encephalopathy species barriers by use of real-time conversion. J Virol. 2015;89(18):9524-9531.

115. de Pedro-Cuesta J, Mahillo-Fernandez I, Rabano A, et al. Nosocomial transmission of sporadic Creutzfeldt-Jakob disease: results from a risk-based assessment of surgical interventions. J Neurol Neurosurg Psychiatry. 2011;82(2):204-212.

116. de Pedro-Cuesta J, Ruiz Tovar M, Ward H, et al. Sensitivity to biases of case-control studies on medical procedures, particularly surgery and blood transfusion, and risk of Creutzfeldt-Jakob disease. Neuroepidemiology. 2012;39(1):1-18.

117. Deleault NR, Geoghegan JC, Nishina K, Kascsak R, Williamson RA, Supattapone S. Protease-resistant prion protein amplification reconstituted with partially purified substrates and synthetic polyanions. J Biol Chem. 2005;280(29):26873-26879.

118. Deleault NR, Harris BT, Rees JR, Supattapone S. Formation of native prions from minimal components *in vitro*. Proc Natl Acad Sci U S A. 2007;104:9741-9746.

119. Dexter E, Kong Q. Neuroprotective effect and potential of cellular prion protein and its cleavage products for treatment of neurodegenerative disorders part I. A literature review. Expert Rev Neurother. 2021;21(9):969-982.

120. Di Gangi S, Bertin M, Noventa M, Cagnin A, Cosmi E, Gizzo S. Obstetric dilemma on the most appropriate management of Creutzfeldt-Jakob disease in pregnancy: seventh case presentation, literature review and new insight. J Matern Fetal Neonatal Med. 2015;28(3):254-261.

121. Diack AB, Alibhai JD, Manson JC. Gene targeted transgenic mouse models in prion research. Prog Mol Biol Transl Sci. 2017;150:157-179.

122. Diack AB, Bartz JC. Experimental models of human prion diseases and prion strains. Handb Clin Neurol. 2018;153:69-84.

123. Douet JY, Huor A, Cassard H, et al. Wide distribution of prion infectivity in the peripheral tissues of vCJD and sCJD patients. Acta Neuropathol. 2021;141(3):383-397.

124. Douet JY, Zafar S, Perret-Liaudet A, et al. Detection of infectivity in blood of persons with variant and sporadic Creutzfeldt-Jakob disease. Emerg Infect Dis. 2014;20(1):114-117.

125. Drisaldi B, Stewart RS, Adles C, et al. Mutant PrP is delayed in its exit from the endoplasmic reticulum, but neither wild-type nor mutant PrP undergoes retrotranslocation prior to proteasomal degradation. J Biol Chem. 2003;278(24):21732-21743.

126. Eisenberg DS, Sawaya MR. Structural studies of amyloid proteins at the molecular level. Annu Rev Biochem. 2017;86:69-95.

127. Ermolayev V, Cathomen T, Merk J, et al. Impaired axonal transport in motor neurons correlates with clinical prion disease. PLoS Pathog. 2009;5(8):e1000558.

128. Ersdal C, Goodsir CM, Simmons MM, McGovern G, Jeffrey M. Abnormal prion protein is associated with changes of plasma membranes and endocytosis in bovine spongiform encephalopathy (BSE)-affected cattle brains. Neuropathol Appl Neurobiol. 2009;35(3):259-271.

129. Escobar LE, Pritzkow S, Winter SN, et al. The ecology of chronic wasting disease in wildlife. Biol Rev Camb Philos Soc. 2020;95(2):393-408.

130. Faris R, Moore RA, Ward A, Sturdevant DE, Priola SA. Mitochondrial respiration is impaired during late-stage hamster prion infection. J Virol. 2017;91(18):e00524-17.

131. Farquhar CF, Dickinson AG. Prolongation of scrapie incubation period by an injection of dextran sulphate 500 within the month before or after infection. J Gen Virol. 1986;67:463-473.

132. Fehlinger A, Wolf H, Hossinger A, et al. Prion strains depend on different endocytic routes for productive infection. Sci Rep. 2017;7(1):6923.

133. Ferreira DG, Temido-Ferreira M, Vicente Miranda H, et al. alpha-Synuclein interacts with PrP(C) to induce cognitive impairment through mGluR5 and NMDAR2B. Nat Neurosci. 2017;20(11):1569-1579.

134. Ferreira NC, Charco JM, Plagenz J, et al. Detection of chronic wasting disease in mule and white-tailed deer by RT-QuIC analysis of outer ear. Sci Rep. 2021;11(1):7702.

135. Ferreira NDC, Caughey B. Proteopathic seed amplification assays for neurodegenerative disorders. Clin Lab Med. 2020;40(3):257-270.

136. Fong JC, Rojas JC, Bang J, et al. Genetic prion disease caused by PRNP Q160X mutation presenting with an orbitofrontal syndrome, cyclic diarrhea, and peripheral neuropathy. J Alzheimers Dis. 2017;55(1):249-258.

137. Foutz A, Appleby BS, Hamlin C, et al. Diagnostic and prognostic value of human prion detection in cerebrospinal fluid. Ann Neurol. 2017;81(1):79-92.

138. Franceschini A, Baiardi S, Hughson AG, et al. High diagnostic value of second generation CSF RT-QuIC across the wide spectrum of CJD prions. Sci Rep. 2017;7(1):10655.

139. Franklin SL, Love S, Greene JR, Betmouni S. Loss of perineuronal net in ME7 prion disease. J Neuropathol Exp Neurol. 2008;67(3):189-199.

140. Gabizon R, Meiner Z, Halimi M, Bensasson SA. Heparin-like molecules bind differentially to prion proteins and change their intracellular metabolic-fate. J Cell Physiol. 1993;157:319-325.

141. Gajdusek DC. Infectious amyloids: subacute spongiform encephalopathies as transmissible cerebral amyloidoses. In: Fields BN, Knipe DM, Howley PM, et al., eds. Fields Virology. 3rd ed. Lippincott-Raven Publishers; 1996:2851-2900.

142. Gallagher-Jones M, Glynn C, Boyer DR, et al. Sub-angstrom cryo-EM structure of a prion protofibril reveals a polar clasp. Nat Struct Mol Biol. 2018;25(2):131-134.

143. Gambetti P, Dong Z, Yuan J, et al. A novel human disease with abnormal prion protein sensitive to protease. Ann Neurol. 2008;63(6):697-708.

144. Gambetti P, Kong Q, Zou W, Parchi P, Chen SG. Sporadic and familial CJD: classification and characterisation. Br Med Bull. 2003;66:213-239.

145. Gerstmann J, Straussler E, Scheinker I. Uber eine eigenartige hereditar-familiare Erkrankung des Zentralnervensystems. Zugleich ein Beitrag zur Frage des vorzeitigen lokalen Alterns. Z Neurol. 1936;154:736-762.

146. Geschwind MD, Murray K. Differential diagnosis with other rapid progressive dementias in human prion diseases. Handb Clin Neurol. 2018;153:371-397.

147. Geschwind MD, Potter CA, Sattavat M, et al. Correlating DWI MRI with pathologic and other features of Jakob-Creutzfeldt disease. Alzheimer Dis Assoc Disord. 2009;23(1):82-87.

148. Geschwind MD, Tan KM, Lennon VA, et al. Voltage-gated potassium channel autoimmunity mimicking Creutzfeldt-Jakob disease. Arch Neurol. 2008;65(10):1341-1346.

149. Ghetti B, Piccardo P, Spillantini MG, et al. Vascular variant of prion protein cerebral amyloidosis with tau-positive neurofibrillary tangles: the phenotype of the stop codon 145 mutation in PRNP. Proc Natl Acad Sci U S A. 1996;93(2):744-748.

150. Ghetti B, Piccardo P, Zanusso G. Dominantly inherited prion protein cerebral amyloidoses—a modern view of Gerstmann-Straussler-Scheinker. Handb Clin Neurol. 2018;153:243-269.

151. Ghetti B, Tagliavini F, Masters CL, et al. Gerstmann-Straussler-Scheinker disease. II. Neurofibrillary tangles and plaques with PrP-amyloid coexist in an affected family. Neurology. 1989;39:1453-1461.

152. Gibbs CJ Jr, Gajdusek DC, Asher DM, et al. Creutzfeldt-Jakob disease (subacute spongiform encephalopathy): transmission to the chimpanzee. Science. 1968;161:388-389.

153. Gill ON, Spencer Y, Richard-Loendt A, et al. Prevalent abnormal prion protein in human appendixes after bovine spongiform encephalopathy epizootic: large scale survey. BMJ. 2013;347:f5675.

154. Gill ON, Spencer Y, Richard-Loendt A, et al. Prevalence in Britain of abnormal prion protein in human appendices before and after exposure to the cattle BSE epizootic. Acta Neuropathol. 2020;139(6):965-976.

155. Glatzel M, Aguzzi A. PrP(C) expression in the peripheral nervous system is a determinant of prion neuroinvasion. J Gen Virol. 2000;81(Pt 11):2813-2821.

156. Glynn C, Sawaya MR, Ge P, et al. Cryo-EM structure of a human prion fibril with a hydrophobic, protease-resistant core. Nat Struct Mol Biol. 2020;27(5):417-423.

157. Godsave SF, Peters PJ, Wille H. Subcellular distribution of the prion protein in sickness and in health. Virus Res. 2015;207:136-145.

158. Godsave SF, Wille H, Pierson J, Prusiner SB, Peters PJ. Plasma membrane invaginations containing clusters of full-length PrPSc are an early form of prion-associated neuropathology in vivo. Neurobiol Aging. 2013;34(6):1621-1631.

159. Goold R, Rabbanian S, Sutton L, et al. Rapid cell-surface prion protein conversion revealed using a novel cell system. Nat Commun. 2011;2:281.

160. Gousset K, Schiff E, Langevin C, et al. Prions hijack tunnelling nanotubes for intercellular spread. Nat Cell Biol. 2009;11(3):328-336.

161. Griffith JS. Self-replication and scrapie. Nature. 1967;215:1043-1044.

162. Groveman BR, Dolan MA, Taubner LM, Kraus A, Wickner RB, Caughey B. Parallel in-register intermolecular beta-sheet architectures for prion-seeded prion protein (PrP) amyloids. J Biol Chem. 2014;289(35):24129-24142.

163. Groveman BR, Raymond GJ, Campbell KJ, et al. Role of the central lysine cluster and scrapie templating in the transmissibility of synthetic prion protein aggregates. PLoS Pathog. 2017;13(9):e1006623.

164. Hadlow WJ. Scrapie and Kuru. Lancet. 1959;2:289-290.

165. Haley N, Donner R, Merrett K, Miller M, Senior K. Selective breeding for disease-resistant PRNP variants to manage chronic wasting disease in farmed whitetail deer. Genes (Basel). 2021;12(9):1396.

166. Haley NJ, Donner R, Henderson DM, et al. Cross-validation of the RT-QuIC assay for the antemortem detection of chronic wasting disease in elk. Prion. 2020;14(1):47-55.

167. Haley NJ, Richt JA, Davenport KA, et al. Design, implementation, and interpretation of amplification studies for prion detection. Prion. 2018;12(2):73-82.

168. Hamaguchi T, Taniguchi Y, Sakai K, et al. Significant association of cadaveric dura mater grafting with subpial Abeta deposition and meningeal amyloid angiopathy. Acta Neuropathol. 2016;132(2):313-315.

169. Hara H, Chida J, Uchiyama K, et al. Neurotropic influenza A virus infection causes prion protein misfolding into infectious prions in neuroblastoma cells. Sci Rep. 2021;11(1):10109.

170. Haraguchi T, Fisher S, Olofsson S, et al. Asparagine-linked glycosylation of the scrapie and cellular prion proteins. Arch Biochem Biophys. 1989;274:1-13.

171. Heidenhain A. Klinische und anatomische Untersuchungen über eine eigenartige organische Erkrankung des Zentralnervensystems im Praesenium. Z Gesamte Neurol Psychiatr. 1929;118:49-111.

172. Heikenwalder M, Zeller N, Seeger H, et al. Chronic lymphocytic inflammation specifies the organ tropism of prions. Science. 2005;307(5712):1107-1110.

173. Hermann P, Appleby B, Brandel JP, et al. Biomarkers and diagnostic guidelines for sporadic Creutzfeldt-Jakob disease. Lancet Neurol. 2021;20(3):235-246.

174. Hill AF, Zeidler M, Ironside J, Collinge J. Diagnosis of new variant Creutzfeldt-Jakob disease by tonsil biopsy. Lancet. 1997;349(9045):99-100.

175. Hilton DA, Ghani AC, Conyers L, et al. Prevalence of lymphoreticular prion protein accumulation in UK tissue samples. J Pathol. 2004;203(3):733-739.

176. Horonchik L, Tzaban S, Ben Zaken O, et al. Heparan sulfate is a cellular receptor for purified infectious prions. J Biol Chem. 2005;280(17):17062-17067.

177. Houston F, Andreoletti O. Animal prion diseases: the risks to human health. Brain Pathol. 2019;29(2):248-262.

178. Hoyt F, Standke HG, Artikis E, et al. Structure of anchorless RML prion reveals motif variation between strains. Nat Commun. 2022;13(1):4005.

179. Hsiao K, Baker HF, Crow TJ, et al. Linkage of a prion protein missense variant to Gerstmann-Straussler syndrome. Nature. 1989;338:342-345.

180. Huor A, Douet JY, Lacroux C, et al. Infectivity in bone marrow from sporadic CJD patients. J Pathol. 2017;243(3):273-278.

181. Irwin DJ, Abrams JY, Schonberger LB, et al. Evaluation of potential infectivity of Alzheimer and Parkinson disease proteins in recipients of cadaver-derived human growth hormone. JAMA Neurol. 2013;70(4):462-468.

182. James TL, Liu H, Ulyanov NB, et al. Solution structure of a 142-residue recombinant prion protein corresponding to the infectious fragment of the scrapie isoform. Proc Natl Acad Sci U S A. 1997;94(19):10086-10091.

183. Jaunmuktane Z, Mead S, Ellis M, et al. Evidence for human transmission of amyloid-beta pathology and cerebral amyloid angiopathy. Nature. 2015;525(7568):247-250.

184. Jaunmuktane Z, Quaegebeur A, Taipa R, et al. Evidence of amyloid-beta cerebral amyloid angiopathy transmission through neurosurgery. Acta Neuropathol. 2018;135(5):671-679.

185. Jeffrey M. Review: membrane-associated misfolded protein propagation in natural transmissible spongiform encephalopathies (TSEs), synthetic prion diseases and Alzheimer's disease. Neuropathol Appl Neurobiol. 2013;39(3):196-216.

186. Jeffrey M, Gonzalez L, Simmons MM, Hunter N, Martin S, McGovern G. Altered trafficking of abnormal prion protein in atypical scrapie: prion protein accumulation in oligodendroglial inner mesaxons. Neuropathol Appl Neurobiol. 2017;43(3):215-226.

187. Jeffrey M, Halliday WG, Bell J, et al. Synapse loss associated with abnormal PrP precedes neuronal degeneration in the scrapie-infected murine hippocampus. Neuropathol Appl Neurobiol. 2000;26(1):41-54.

188. Jeffrey M, McGovern G, Goodsir CM, Siso S, Gonzalez L. Strain-associated variations in abnormal PrP trafficking of sheep scrapie. Brain Pathol. 2009;19(1):1-11.

189. Jeffrey M, McGovern G, Siso S, Gonzalez L. Cellular and sub-cellular pathology of animal prion diseases: relationship between morphological changes, accumulation of abnormal prion protein and clinical disease. Acta Neuropathol. 2011;121(1):113-134.

190. Jing D, Chen Y, Xie K, et al. White matter integrity involvement in the preclinical stage of familial Creutzfeldt-Jakob disease: a diffusion tensor imaging study. Front Aging Neurosci. 2021;13:655667.

191. Jones E, Hummerich H, Vire E, et al. Identification of novel risk loci and causal insights for sporadic Creutzfeldt-Jakob disease: a genome-wide association study. Lancet Neurol. 2020;19(10):840-848.

192. Kanu N, Imokawa Y, Drechsel DN, et al. Transfer of scrapie prion infectivity by cell contact in culture. Curr Biol. 2002;12(7):523-530.

193. Kaski D, Mead S, Hyare H, et al. Variant CJD in an individual heterozygous for PRNP codon 129. Lancet. 2009;374(9707):2128.

194. Katscher F. It's Jakob's disease, not Creutzfeldt's. Nature. 1998;393(6680):11.

195. Kim JI, Cali I, Surewicz K, et al. Mammalian prions generated from bacterially expressed prion protein in the absence of any mammalian cofactors. J Biol Chem. 2010;285(19):14083-14087.

196. Kimberlin RH, Cole S, Walker CA. Temporary and permanent modifications to a single strain of mouse scrapie on transmission to rats and hamsters. J Gen Virol. 1987;68:1875-1881.

197. Kimberlin RH, Walker CA. Competition between strains of scrapie depends on the blocking agent being infectious. Intervirology. 1985;23:74-81.

198. Kimberlin RH, Walker CA. Suppression of scrapie infection in mice by heteropolyanion 23, dextran sulfate, and some other polyanions. Antimicrob Agents Chemother. 1986;30:409-413.

199. Kimberlin RH, Walker CA. Pathogenesis of experimental scrapie. Ciba Found Symp. 1988;135:37-62.

200. Klingeborn M, Race B, Meade-White KD, Rosenke R, Striebel JF, Chesebro B. Crucial role for prion protein membrane anchoring in the neuroinvasion and neural spread of prion infection. J Virol. 2011;85(4):1484-1494.

201. Klohn PC, Stoltze L, Flechsig E, Enari M, Weissmann C. A quantitative, highly sensitive cell-based infectivity assay for mouse scrapie prions. Proc Natl Acad Sci U S A. 2003;100(20):11666-11671.

202. Kobayashi A, Parchi P, Yamada M, Mohri S, Kitamoto T. Neuropathological and biochemical criteria to identify acquired Creutzfeldt-Jakob disease among presumed sporadic cases. Neuropathology. 2016;36(3):305-310.

203. Kocisko DA, Baron GS, Rubenstein R, Chen J, Kuizon S, Caughey B. New inhibitors of scrapie-associated prion protein formation in a library of 2000 drugs and natural products. J Virol. 2003;77(19):10288-10294.

204. Kocisko DA, Come JH, Priola SA, et al. Cell-free formation of protease-resistant prion protein. Nature. 1994;370:471-474.

205. Kocisko DA, Priola SA, Raymond GJ, Chesebro B, Lansbury PT Jr, Caughey B. Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier. Proc Natl Acad Sci USA. 1995;92:3923-3927.

206. Kong Q, Huang S, Zou W, et al. Chronic wasting disease of elk: transmissibility to humans examined by transgenic mouse models. J Neurosci. 2005;25(35):7944-7949.

207. Konold T, Arnold ME, Austin AR, et al. Bovine spongiform encephalopathy: the effect of oral exposure dose on attack rate and incubation period in cattle—an update. BMC Res Notes. 2012;5:674.

208. Konold T, Bone GE, Clifford D, et al. Experimental H-type and L-type bovine spongiform encephalopathy in cattle: observation of two clinical syndromes and diagnostic challenges. BMC Vet Res. 2012;8:22.

209. Kovacs GG, Puopolo M, Ladogana A, et al. Genetic prion disease: the EUROCJD experience. Hum Genet. 2005;118(2):166-174.

210. Krasnianski A, Sanchez Juan P, Ponto C, et al. A proposal of new diagnostic pathway for fatal familial insomnia. J Neurol Neurosurg Psychiatry. 2014;85(6):654-659.

211. Kraus A, Groveman BR, Caughey B. Prions and the potential transmissibility of protein misfolding diseases. Annu Rev Microbiol. 2013;67:543-564.

212. Kraus A, Hoyt F, Schwartz CL, et al. High-resolution structure and strain comparison of infectious mammalian prions. Mol Cell. 2021;81(21):4540-4551.

213. Kraus A, Raymond GJ, Race B, et al. PrP P102L and nearby lysine mutations promote spontaneous in vitro formation of transmissible prions. J Virol. 2017;91(21):e01276.

214. Kuffer A, Lakkaraju AK, Mogha A, et al. The prion protein is an agonistic ligand of the G protein-coupled receptor Adgrg6. Nature. 2016;536(7617):464-468.

215. Kumagai S, Daikai T, Onodera T. Bovine spongiform encephalopathy—a review from the perspective of food safety. Food Saf (Tokyo). 2019;7(2):21-47.

216. Kuznetsova A, McKenzie D, Cullingham C, Aiken JM. Long-term incubation PrP(CWD) with soils affects prion recovery but not infectivity. Pathogens. 2020;9(4):311.

217. Ladogana A, Puopolo M, Croes EA, et al. Mortality from Creutzfeldt-Jakob disease and related disorders in Europe, Australia, and Canada. Neurology. 2005;64(9):1586-1591.

218. Lane KL, Brown P, Howell DN, et al. Creutzfeldt-Jakob disease in a pregnant woman with an implanted dura mater graft. Neurosurgery. 1994;34(4):737-739; discussion 739-740.

219. Lattanzio F, Abu-Rumeileh S, Franceschini A, et al. Prion-specific and surrogate CSF biomarkers in Creutzfeldt-Jakob disease: diagnostic accuracy in relation to molecular subtypes and analysis of neuropathological correlates of p-tau and Abeta42 levels. Acta Neuropathol. 2017;133(4):559-578.

220. Lauren J, Gimbel DA, Nygaard HB, Gilbert JW, Strittmatter SM. Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. Nature. 2009;457(7233):1128-1132.

221. Le NTT, Wu B, Harris DA. Prion neurotoxicity. Brain Pathol. 2019;29(2):263-277.

222. Leblanc P, Alais S, Porto-Carreiro I, et al. Retrovirus infection strongly enhances scrapie infectivity release in cell culture. EMBO J. 2006;25(12):2674-2685.

223. Legname G, Baskakov IV, Nguyen HO, et al. Synthetic mammalian prions. Science. 2004;305(5684):673-676.

224. Leitao MJ, Baldeiras I, Almeida MR, et al. Sporadic Creutzfeldt-Jakob disease diagnostic accuracy is improved by a new CSF ELISA 14-3-3gamma assay. Neuroscience. 2016;322:398-407.

225. Li C, Wang D, Wu W, et al. DLP1-dependent mitochondrial fragmentation and redistribution mediate prion-associated mitochondrial dysfunction and neuronal death. Aging Cell. 2018;17(1):e12693.

226. Li Q, Wang F, Xiao X, et al. Structural attributes of mammalian prion infectivity: Insights from studies with synthetic prions. J Biol Chem. 2018;293(48):18494-18503.

227. Liang J, Wang W, Sorensen D, et al. Cellular prion protein regulates its own alpha-cleavage through ADAM8 in skeletal muscle. J Biol Chem. 2012;287(20):16510-16520.

228. Liberski PP. Subacute spongiform encephalopathies—the transmissible brain amyloidoses: a comparison with the non-transmissible brain amyloidoses of Alzheimer type. J Comp Pathol. 1993;109(2):103-127.

229. Liberski PP, Gajos A, Sikorska B, Lindenbaum S. Kuru, the first human prion disease. Viruses. 2019;11(3):232.

230. Liberski PP, Sikorska B, Brown P. Kuru: the first prion disease. Adv Exp Med Biol 2012;724:143-153.

231. Linden R. The biological function of the prion protein: a cell surface scaffold of signaling modules. Front Mol Neurosci. 2017;10:77.

232. Linsenmeier L, Mohammadi B, Shafiq M, et al. Ligands binding to the prion protein induce its proteolytic release with therapeutic potential in neurodegenerative proteinopathies. Sci Adv. 2021;7(48):eabj1826.

233. Liu S, Hossinger A, Heumuller SE, et al. Highly efficient intercellular spreading of protein misfolding mediated by viral ligand-receptor interactions. Nat Commun. 2021;12(1):5739.

234. Llorens F, Schmitz M, Karch A, et al. Comparative analysis of cerebrospinal fluid biomarkers in the differential diagnosis of neurodegenerative dementia. Alzheimers Dement. 2016;12(5):577-589.

235. Locht C, Chesebro B, Race R, Keith JM. Molecular cloning and complete sequence of prion protein cDNA from mouse brain infected with the scrapie agent. Proc Natl Acad Sci U S A. 1986;83:6372-6376.

236. Lombardi G, Casalone C, D'Angelo A, et al. Intraspecies transmission of BASE induces clinical dullness and amyotrophic changes. PLoS Pathog. 2008;4(5):e1000075.

237. Lopez FJG, Ruiz-Tovar M, Almazan-Isla J, Alcalde-Cabero E, Calero M, de Pedro-Cuesta J. Risk of transmission of sporadic Creutzfeldt-Jakob disease by surgical procedures: systematic reviews and quality of evidence. Euro Surveill. 2017;22(43):16-00806.

238. Lu H, Jing D, Chen Y, et al. Metabolic changes detected by 18F-FDG PET in the preclinical stage of familial Creutzfeldt-Jakob disease. J Alzheimers Dis. 2020;77(4):1513-1521.

239. Lugaresi E, Medori R, Montagna P, et al. Fatal familial insomnia and dysautonomia with selective degeneration of thalamic nuclei. N Engl J Med. 1986;315:997-1003.

240. Luk C, Jones S, Thomas C, et al. Diagnosing sporadic Creutzfeldt-Jakob disease by the detection of abnormal prion protein in patient urine. JAMA Neurol. 2016;73(12):1454-1460.

241. Luk CC, Mathiason CK, Orru CD, et al. Creutzfeldt-Jakob disease in pregnancy: the use of modified RT-QuIC to determine infectivity in placental tissues. Prion. 2021;15(1):107-111.

242. Ma Y, Ma J. Immunotherapy against prion disease. Pathogens. 2020;9(3):216.

243. Mabbott NA. Immunology of prion protein and prions. Prog Mol Biol Transl Sci. 2017;150:203-240.

244. Mabbott NA, Alibhai JD, Manson J. The role of the immune system in prion infection. Handb Clin Neurol. 2018;153:85-107.

245. Magalhaes AC, Baron GS, Lee KS, et al. Uptake and neuritic transport of scrapie prion protein coincident with infection of neuronal cells. J Neurosci. 2005;25(21):5207-5216.

246. Makarava N, Baskakov IV. The evolution of transmissible prions: the role of deformed templating. PLoS Pathog. 2013;9(12):e1003759.

247. Makarava N, Chang JC, Molesworth K, Baskakov IV. Posttranslational modifications define course of prion strain adaptation and disease phenotype. J Clin Invest. 2020;130(8):4382-4395.

248. Makarava N, Kovacs GG, Bocharova O, et al. Recombinant prion protein induces a new transmissible prion disease in wild-type animals. Acta Neuropathol. 2010;119(2):177-187.

249. Mallucci G, Dickinson A, Linehan J, Klohn PC, Brandner S, Collinge J. Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis. Science. 2003;302(5646):871-874.

250. Mammana A, Baiardi S, Rossi M, et al. Detection of prions in skin punch biopsies of Creutzfeldt-Jakob disease patients. Ann Clin Transl Neurol. 2020;7(4):559-564.

251. Manetto V, Medori R, Cortelli P, et al. Fatal familial insomnia: clinical and pathologic study of five new cases. Neurology. 1992;42(2):312-319.

252. Mange A, Beranger F, Peoc'h K, Onodera T, Frobert Y, Lehmann S. Alpha- and beta- cleavages of the amino-terminus of the cellular prion protein. Biol Cell. 2004;96(2):125-132.

253. Manka SW, Zhang W, Wenborn A, et al. 2.7 Å cryo-EM structure of ex vivo RML prion fibrils. Nat Commun. 2022;13(1):4004.

254. Manson J, West JD, Thomson V, McBride P, Kaufman MH, Hope J. The prion protein gene: a role in mouse embryogenesis? Development. 1992;115:117-122.

255. Marella M, Chabry J. Neurons and astrocytes respond to prion infection by inducing microglia recruitment. J Neurosci. 2004;24(3):620-627.

256. Marijanovic Z, Caputo A, Campana V, Zurzolo C. Identification of an intracellular site of prion conversion. PLoS Pathog. 2009;5(5):e1000426.

257. Marin-Moreno A, Aguilar-Calvo P, Espinosa JC, et al. Classical scrapie in small ruminants is caused by at least four different prion strains. Vet Res. 2021;52(1):57.

258. Marin B, Otero A, Lugan S, et al. Classical BSE prions emerge from asymptomatic pigs challenged with atypical/Nor98 scrapie. Sci Rep. 2021;11(1):17428.

259. Marsh RF, Kincaid AE, Bessen RA, Bartz JC. Interspecies transmission of chronic wasting disease prions to squirrel monkeys (Saimiri sciureus). J Virol. 2005;79(21):13794-13796.

260. Mathiason CK, Powers JG, Dahmes SJ, et al. Infectious prions in the saliva and blood of deer with chronic wasting disease. Science. 2006;314(5796):133-136.

261. Mays CE, van der Merwe J, Kim C, et al. Prion infectivity plateaus and conversion to symptomatic disease originate from falling precursor levels and increased levels of oligomeric PrPSc species. J Virol. 2015;89(24):12418-12426.

262. McAlary L, Chew YL, Lum JS, Geraghty NJ, Yerbury JJ, Cashman NR. Amyotrophic lateral sclerosis: proteins, proteostasis, prions, and promises. Front Cell Neurosci. 2020;14:581907.

263. McKinnon C, Goold R, Andre R, et al. Prion-mediated neurodegeneration is associated with early impairment of the ubiquitin-proteasome system. Acta Neuropathol. 2016;131(3):411-425.

264. McLennan NF, Brennan PM, McNeill A, et al. Prion protein accumulation and neuroprotection in hypoxic brain damage. Am J Pathol. 2004;165(1):227-235.

265. McNulty E, Nalls AV, Mellentine S, et al. Comparison of conventional, amplification and bio-assay detection methods for a chronic wasting disease inoculum pool. PLoS One. 2019;14(5):e0216621.

266. Mead S, Gandhi S, Beck J, et al. A novel prion disease associated with diarrhea and autonomic neuropathy. N Engl J Med. 2013;369(20):1904-1914.

267. Mead S, Lloyd S, Collinge J. Genetic factors in mammalian prion diseases. Annu Rev Genet. 2019;53:117-147.

268. Mead S, Poulter M, Uphill J, et al. Genetic risk factors for variant Creutzfeldt-Jakob disease: a genome-wide association study. Lancet Neurol. 2009;8(1):57-66.

269. Mead S, Whitfield J, Poulter M, et al. A novel protective prion protein variant that colocalizes with kuru exposure. N Engl J Med. 2009;361(21):2056-2065.

270. Medori R, Tritschler HJ, LeBlanc A, et al. Fatal familial insomnia, a prion disease with a mutation at codon 178 of the prion protein gene. N Engl J Med. 1992;326(7):444-449.

271. Meisl G, Kurt T, Condado-Morales I, et al. Scaling analysis reveals the mechanism and rates of prion replication in vivo. Nat Struct Mol Biol. 2021;28(4):365-372.

272. Mente KP, O'Donnell JK, Jones SE, et al. Fluorodeoxyglucose Positron Emission Tomography (FDG-PET) correlation of histopathology and MRI in prion disease. Alzheimer Dis Assoc Disord. 2017;31(1):1-7.

273. Merz PA, Somerville RA, Wisniewski HM, Iqbal K. Abnormal fibrils from scrapie-infected brain. Acta Neuropathol. 1981;54:63-74.

274. Minikel EV, Karczewski KJ, Martin HC, et al. Evaluating drug targets through human loss-of-function genetic variation. Nature. 2020;581(7809):459-464.

275. Minikel EV, Vallabh SM, Lek M, et al. Quantifying prion disease penetrance using large population control cohorts. Sci Transl Med. 2016;8(322):322ra9.

276. Minikel EV, Vallabh SM, Orseth MC, et al. Age at onset in genetic prion disease and the design of preventive clinical trials. Neurology. 2019;93(2):e125-e134.

277. Minikel EV, Zhao HT, Le J, et al. Prion protein lowering is a disease-modifying therapy across prion disease stages, strains and endpoints. Nucleic Acids Res. 2020;48(19):10615-10631.

278. Moazami-Goudarzi K, Andreoletti O, Vilotte JL, Beringue V. Review on PRNP genetics and susceptibility to chronic wasting disease of Cervidae. Vet Res. 2021;52(1):128.

279. Moda F, Suardi S, Di Fede G, et al. MM2-thalamic Creutzfeldt-Jakob disease: neuropathological, biochemical and transmission studies identify a distinctive prion strain. Brain Pathol. 2012;22(5):662-669.

280. Moreno JA, Radford H, Peretti D, et al. Sustained translational repression by eIF2alpha-P mediates prion neurodegeneration. Nature. 2012;485(7399):507-511.

281. Myers R, Cembran A, Fernandez-Funez P. Insight from animals resistant to prion diseases: deciphering the genotype - morphotype - phenotype code for the prion protein. Front Cell Neurosci. 2020;14:254.

282. Nalls AV, McNulty E, Powers J, et al. Mother to offspring transmission of chronic wasting disease in reeves' muntjac deer. PLoS One. 2013;8(8):e71844.

283. Naslavsky N, Stein R, Yanai A, Friedlander G, Taraboulos A. Characterization of detergent-insoluble complexes containing the cellular prion protein and its scrapie isoform. J Biol Chem. 1997;272(10):6324-6331.

284. Nazor Friberg K, Hung G, Wancewicz E, et al. Intracerebral infusion of antisense oligonucleotides into prion-infected mice. Mol Ther Nucleic Acids. 2012;1:e9.

285. Nemani SK, Myskiw JL, Lamoureux L, Booth SA, Sim VL. Exposure risk of chronic wasting disease in humans. Viruses. 2020;12(12):1454.

286. Noble GP, Wang DW, Walsh DJ, et al. A structural and functional comparison between infectious and non-infectious autocatalytic recombinant PrP conformers. PLoS Pathog. 2015;11(6):e1005017.

287. Nonno R, Di Bari MA, Cardone F, et al. Efficient transmission and characterization of Creutzfeldt-Jakob disease strains in bank voles. PLoS Pathog. 2006;2(2):e12.

288. Notari S, Appleby BS, Gambetti P. Variably protease-sensitive prionopathy. Handb Clin Neurol. 2018;153:175-190.

289. Oesch B, Westaway D, Walchli M, et al. A cellular gene encodes scrapie PrP 27-30 protein. Cell. 1985;40:735-746.

290. Okada H, Iwamaru Y, Imamura M, et al. Oral transmission of L-type bovine spongiform encephalopathy agent among cattle. Emerg Infect Dis. 2017;23(2):284-287.

291. Ondrejcak T, Klyubin I, Corbett GT, et al. Cellular prion protein mediates the disruption of hippocampal synaptic plasticity by soluble tau in vivo. J Neurosci. 2018;38(50):10595-10606.

292. Orru CD, Bongianni M, Tonoli G, et al. A test for Creutzfeldt-Jakob disease using nasal brushings. N Engl J Med. 2014;371(6):519-529.

293. Orru CD, Groveman BR, Foutz A, et al. Ring trial of 2nd generation RT-QuIC diagnostic tests for sporadic CJD. Ann Clin Transl Neurol. 2020;7(11):2262-2271.

294. Orru CD, Groveman BR, Raymond LD, et al. Bank vole prion protein as an apparently universal substrate for RT-QuIC-based detection and discrimination of prion strains. PLoS Pathog. 2015;11(6):e1004983.

295. Orru CD, Soldau K, Cordano C, et al. Prion seeds distribute throughout the eyes of sporadic Creutzfeldt-Jakob disease patients. MBio. 2018;9(6):e02095-18.

296. Orru CD, Wilham JM, Raymond LD, et al. Prion disease blood test using immunoprecipitation and improved quaking-induced conversion. MBio. 2011;2(3):e00078-11.

297. Orru CD, Yuan J, Appleby BS, et al. Prion seeding activity and infectivity in skin samples from patients with sporadic Creutzfeldt-Jakob disease. Sci Transl Med. 2017;9(417):eaam7785.

298. Ortega-Cubero S, Luquin MR, Dominguez I, et al. Structural and functional neuroimaging in human prion diseases. Neurologia. 2013;28(5):299-308.

299. Ostapchenko VG, Beraldo FH, Mohammad AH, et al. The prion protein ligand, stress-inducible phosphoprotein 1, regulates amyloid-beta oligomer toxicity. J Neurosci. 2013;33(42):16552-16564.

300. Pan K-M, Baldwin M, Nguyen J, et al. Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion protein. Proc Natl Acad Sci U S A. 1993;90:10962-10966.

301. Parchi P, Chen SG, Brown P, et al. Different patterns of truncated prion protein fragments correlate with distinct phenotypes in P102L Gerstmann-Straussler-Scheinker disease. Proc Natl Acad Sci U S A. 1998;95(14):8322-8327.

302. Parchi P, Giese A, Capellari S, et al. Classification of sporadic Creutzfeldt-Jakob disease based on molecular and phenotypic analysis of 300 subjects. Ann Neurol. 1999;46(2):224-233.

303. Parchi P, Strammiello R, Notari S, et al. Incidence and spectrum of sporadic Creutzfeldt-Jakob disease variants with mixed phenotype and co-occurrence of PrPSc types: an updated classification. Acta Neuropathol. 2009;118(5):659-671.

304. Pascuzzo R, Oxtoby NP, Young AL, et al. Prion propagation estimated from brain diffusion MRI is subtype dependent in sporadic Creutzfeldt-Jakob disease. Acta Neuropathol. 2020;140(2):169-181.

305. Pattison IH, Jones KM. The possible nature of the transmissible agent of scrapie. Vet Rec. 1967;80:2-9.

306. Petit-Paitel A, Menard B, Guyon A, et al. Prion protein is a key determinant of alcohol sensitivity through the modulation of *N*-methyl-D-aspartate receptor (NMDAR) activity. PLoS One. 2012;7(4):e34691.

307. Piccardo P, Dlouhy SR, Lievens PM, et al. Phenotypic variability of Gerstmann-Straussler-Scheinker disease is associated with prion protein heterogeneity. J Neuropathol Exp Neurol. 1998;57(10):979-988.

308. Pocchiari M, Puopolo M, Croes EA, et al. Predictors of survival in sporadic Creutzfeldt-Jakob disease and other human transmissible spongiform encephalopathies. Brain. 2004;127(Pt 10):2348-2359.

309. Priola SA. Cell biology of prion infection. Handb Clin Neurol. 2018;153:45-68.

310. Priola SA, Chabry J, Chan K. Efficient conversion of normal prion protein (PrP) by abnormal hamster PrP is determined by homology at amino acid residue 155. J Virol. 2001;75(10):4673-4680.

311. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science. 1982;216:136-144.

312. Prusiner SB. Prions. Proc Natl Acad Sci U S A. 1998;95(23):13363-13383.

313. Prusiner SB. Prions. In: Knipe DM, Howley P, eds. Fields Virology. Vol 2. 6th ed. Wolters Kluwer Health; 2013.

314. Prusiner SB, Groth D, Serban A, et al. Ablation of the prion protein (PrP) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies. Proc Natl Acad Sci U S A. 1993;90(22):10608-10612.

315. Prusiner SB, Groth DF, Bolton DC, Kent SB, Hood LE. Purification and structural studies of a major scrapie prion protein. Cell. 1984;38:127-134.

316. Puoti G, Bizzi A, Forloni G, Safar JG, Tagliavini F, Gambetti P. Sporadic human prion diseases: molecular insights and diagnosis. Lancet Neurol. 2012;11(7):618-628.

317. Qina T, Sanjo N, Hizume M, et al. Clinical features of genetic Creutzfeldt-Jakob disease with V180I mutation in the prion protein gene. BMJ Open. 2014;4(5):e004968.

318. Race B, Meade-White KD, Miller MW, et al. Susceptibilities of nonhuman primates to chronic wasting disease. Emerg Infect Dis. 2009;15(9):1366-1376.

319. Race B, Williams K, Chesebro B. Transmission studies of chronic wasting disease to transgenic mice overexpressing human prion protein using the RT-QuIC assay. Vet Res. 2019;50(1):6.

320. Race B, Williams K, Hughson AG, et al. Familial human prion diseases associated with prion protein mutations Y226X and G131V are transmissible to transgenic mice expressing human prion protein. Acta Neuropathol Commun. 2018;6(1):13.

321. Race B, Williams K, Orru CD, Hughson AG, Lubke L, Chesebro B. Lack of transmission of chronic wasting disease to cynomolgus Macaques. J Virol. 2018;92(14):e00550-18.

322. Race R, Raines A, Raymond GL, Caughey B, Chesebro B. Long-term subclinical carrier state precedes scrapie replication and adaptation in a resistant species: analogies to bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease in humans. J Virol. 2001;75(21):10106-10112.

323. Rambold AS, Muller V, Ron U, Ben-Tal N, Winklhofer KF, Tatzelt J. Stress-protective signalling of prion protein is corrupted by scrapie prions. EMBO J. 2008;27(14):1974-1984.

324. Raymond GJ, Hope J, Kocisko DA, et al. Molecular assessment of the transmissibilities of BSE and scrapie to humans. Nature. 1997;388:285-288.

325. Raymond GJ, Race B, Orru CD, et al. Transmission of CJD from nasal brushings but not spinal fluid or RT-QuIC product. Ann Clin Transl Neurol. 2020;7(6):932-944.

326. Raymond GJ, Zhao HT, Race B, et al. Antisense oligonucleotides extend survival of prion-infected mice. JCI Insight. 2019;5(16):e131175.

327. Relano-Gines A, Lehmann S, Crozet C. Cell-based therapy against prion diseases. Curr Opin Pharmacol. 2019;44:8-14.

328. Resenberger UK, Harmeier A, Woerner AC, et al. The cellular prion protein mediates neurotoxic signalling of beta-sheet-rich conformers independent of prion replication. EMBO J. 2011;30(10):2057-2070.

329. Richt JA, Kasinathan P, Hamir AN, et al. Production of cattle lacking prion protein. Nat Biotechnol. 2007;25(1):132-138.

330. Riemenschneider M, Wagenpfeil S, Vanderstichele H, et al. Phospho-tau/total tau ratio in cerebrospinal fluid discriminates Creutzfeldt-Jakob disease from other dementias. Mol Psychiatry. 2003;8(3):343-347.

331. Rouvinski A, Karniely S, Kounin M, et al. Live imaging of prions reveals nascent PrPSc in cell-surface, raft-associated amyloid strings and webs. J Cell Biol. 2014;204(3):423-441.

332. S-Juan P, Ward HJ, De Silva R, Knight RS, Will RG. Ophthalmic surgery and Creutzfeldt-Jakob disease. Br J Ophthalmol. 2004;88(4):446-449.

333. Saa P, Castilla J, Soto C. Presymptomatic detection of prions in blood. Science. 2006;313(5783):92-94.

334. Saborio GP, Permanne B, Soto C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature. 2001;411(6839):810-813.

335. Safar J, Wille H, Itri V, et al. Eight prion strains have PrP(Sc) molecules with different conformations [see comments]. Nat Med. 1998;4(10):1157-1165.

336. Safar JG, DeArmond SJ, Kociuba K, et al. Prion clearance in bigenic mice. J Gen Virol. 2005;86(Pt 10):2913-2923.

337. Sajnani G, Silva CJ, Ramos A, et al. PK-sensitive PrP is infectious and shares basic structural features with PK-resistant PrP. PLoS Pathog. 2012;8(3):e1002547.

338. Sandberg MK, Al-Doujaily H, Sigurdson CJ, et al. Chronic wasting disease prions are not transmissible to transgenic mice overexpressing human prion protein. J Gen Virol. 2010;91(Pt 10):2651-2657.

339. Sano K, Satoh K, Atarashi R, et al. Early detection of abnormal prion protein in genetic human prion diseases now possible using real-time QUIC assay. PLoS One. 2013;8(1):e54915.

340. Santuccione A, Sytnyk V, Leshchyns'ka I, Schachner M. Prion protein recruits its neuronal receptor NCAM to lipid rafts to activate p59fyn and to enhance neurite outgrowth. J Cell Biol. 2005;169(2):341-354.

341. Sarnataro D, Pepe A, Zurzolo C. Cell biology of prion protein. Prog Mol Biol Transl Sci. 2017;150:57-82.

342. Scheiblich H, Dansokho C, Mercan D, et al. Microglia jointly degrade fibrillar alpha-synuclein cargo by distribution through tunneling nanotubes. Cell. 2021;184(20):5089-5106 e21.

343. Schmitt-Ulms G, Legname G, Baldwin MA, et al. Binding of neural cell adhesion molecules (N-CAMs) to the cellular prion protein. J Mol Biol. 2001;314(5):1209-1225.

344. Schmitz M, Ebert E, Stoeck K, et al. Validation of 14-3-3 protein as a marker in sporadic Creutzfeldt-Jakob disease diagnostic. Mol Neurobiol. 2016;53(4):2189-2199.

345. Schneider B, Baudry A, Pietri M, et al. The cellular prion protein-ROCK connection: contribution to neuronal homeostasis and neurodegenerative diseases. Front Cell Neurosci. 2021;15:660683.

346. Scott M, Groth D, Foster D, et al. Propagation of prions with artificial properties in transgenic mice expressing chimeric PrP genes. Cell. 1993;73:979-988.

347. Selariu A, Powers JG, Nalls A, et al. In utero transmission and tissue distribution of chronic wasting disease-associated prions in free-ranging Rocky Mountain elk. J Gen Virol. 2015;96(11):3444-3455.

348. Sevillano AM, Aguilar-Calvo P, Kurt TD, et al. Prion protein glycans reduce intracerebral fibril formation and spongiosis in prion disease. J Clin Invest. 2020;130(3):1350-1362.

349. Shearin H, Bessen RA. Axonal and transynaptic spread of prions. J Virol. 2014;88(15):8640-8655.

350. Shibuya S, Higuchi J, Shin RW, Tateishi J, Kitamoto T. Protective prion protein polymorphisms against sporadic Creutzfeldt-Jakob disease. Lancet. 1998;351(9100):419.

351. Siddiqi MK, Kim C, Haldiman T, et al. Structurally distinct external solvent-exposed domains drive replication of major human prions. PLoS Pathog. 2021;17(6):e1009642.

352. Sigurdson CJ, Bartz JC, Glatzel M. Cellular and molecular mechanisms of prion disease. Annu Rev Pathol. 2019;14:497-516.

353. Sigurdson CJ, Miller MW. Other animal prion diseases. Br Med Bull. 2003;66:199-212.

354. Sikorska B, Liberski PP, Brown P. Neuronal autophagy and aggresomes constitute a consistent part of neurodegeneration in experimental scrapie. Folia Neuropathol. 2007;45(4):170-178.

355. Silveira JR, Hughson AG, Caughey B. Fractionation of prion protein aggregates by asymmetrical flow field-flow fractionation. Methods Enzymol. 2006;412:21-33.

356. Silveira JR, Raymond GJ, Hughson AG, et al. The most infectious prion protein particles. Nature. 2005;437(7056):257-261.

357. Sim VL. Prion disease: chemotherapeutic strategies. Infect Disord Drug Targets. 2012;12(2):144-160.

358. Sim VL. Prion diseases. In: Jackson AC, ed. Viral Infections of the Human Nervous System. Springer; 2013:371-401.

359. Skillback T, Rosen C, Asztely F, Mattsson N, Blennow K, Zetterberg H. Diagnostic performance of cerebrospinal fluid total tau and phosphorylated tau in Creutzfeldt-Jakob disease: results from the Swedish Mortality Registry. JAMA Neurol. 2014;71(4):476-483.

360. Smirnovas V, Baron GS, Offerdahl DK, Raymond GJ, Caughey B, Surewicz WK. Structural organization of brain-derived mammalian prions examined by hydrogen-deuterium exchange. Nat Struct Mol Biol. 2011;18(4):504-506.

361. Soto C. Unfolding the role of protein misfolding in neurodegenerative diseases. Nat Rev Neurosci. 2003;4(1):49-60.

362. Spagnolli G, Rigoli M, Orioli S, et al. Full atomistic model of prion structure and conversion. PLoS Pathog. 2019;15(7):e1007864.

363. Stahl N, Baldwin MA, Burlingame AL, Prusiner SB. Identification of glycoinositol phospholipid linked and truncated forms of the scrapie prion protein. Biochemistry. 1990;29:8879-8884.

364. Stahl N, Borchelt DR, Hsiao K, Prusiner SB. Scrapie prion protein contains a phosphatidylinositol glycolipid. Cell. 1987;51:229-240.

365. Steele AD, Emsley JG, Ozdinler PH, Lindquist S, Macklis JD. Prion protein (PrPc) positively regulates neural precursor proliferation during developmental and adult mammalian neurogenesis. Proc Natl Acad Sci U S A. 2006;103(9):3416-3421.

366. Steele AD, Lindquist S, Aguzzi A. The prion protein knockout mouse: a phenotype under challenge. Prion. 2007;1(2):83-93.

367. Steinhoff BJ, Zerr I, Glatting M, Schulz-Schaeffer W, Poser S, Kretzschmar HA. Diagnostic value of periodic complexes in Creutzfeldt-Jakob disease. Ann Neurol. 2004;56(5):702-708.

368. Stoeck K, Sanchez-Juan P, Gawinecka J, et al. Cerebrospinal fluid biomarker supported diagnosis of Creutzfeldt-Jakob disease and rapid dementias: a longitudinal multicentre study over 10 years. Brain. 2012;135(Pt 10):3051-3061.

369. Striebel JF, Race B, Chesebro B. Prion protein and susceptibility to kainate-induced seizures: genetic pitfalls in the use of PrP knockout mice. Prion. 2013;7(4):280-285.

370. Striebel JF, Race B, Leung JM, Schwartz C, Chesebro B. Prion-induced photoreceptor degeneration begins with misfolded prion protein accumulation in cones at two distinct sites: cilia and ribbon synapses. Acta Neuropathol Commun. 2021;9(1):17.

371. Supattapone S. Cofactor molecules: essential partners for infectious prions. Prog Mol Biol Transl Sci. 2020;175:53-75.

372. Tamai Y, Kojima H, Kitajima R, et al. Demonstration of the transmissible agent in tissue from a pregnant woman with Creutzfeldt-Jakob disease. N Engl J Med. 1992; 327(9):649.

373. Tamguney G, Francis KP, Giles K, Lemus A, DeArmond SJ, Prusiner SB. Measuring prions by bioluminescence imaging. Proc Natl Acad Sci U S A. 2009;106(35):15002-15006.

374. Taraboulos A, Raeber AJ, Borchelt DR, Serban D, Prusiner SB. Synthesis and trafficking of prion proteins in cultured cells. Mol Biol Cell. 1992;3:851-863.

375. Tateishi J, Brown P, Kitamoto T, et al. First experimental transmission of fatal familial insomnia. Nature. 1995;376:434-435.

376. Taylor DR, Parkin ET, Cocklin SL, et al. Role of ADAMs in the ectodomain shedding and conformational conversion of the prion protein. J Biol Chem. 2009;284(34):22590-22600.

377. Telling GC, Parchi P, DeArmond SJ, et al. Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity. Science. 1996;274:2079-2082.

378. Teruya K, Doh-Ura K. Insights from therapeutic studies for PrP prion disease. Cold Spring Harb Perspect Med. 2017;7(3):a024430.

379. Tesar A, Matej R, Kukal J, et al. Clinical variability in P102L Gerstmann-Straussler-Scheinker syndrome. Ann Neurol. 2019;86(5):643-652.

380. Theint T, Nadaud PS, Aucoin D, et al. Species-dependent structural polymorphism of Y145Stop prion protein amyloid revealed by solid-state NMR spectroscopy. Nat Commun. 2017;8(1):753.

381. Thom T, Schmitz M, Fischer AL, et al. Cellular prion protein mediates alpha-synuclein uptake, localization, and toxicity in vitro and in vivo. Mov Disord. 2021;37(1):39-51.

382. Thompson AGB, Anastasiadis P, Druyeh R, et al. Evaluation of plasma tau and neurofilament light chain biomarkers in a 12-year clinical cohort of human prion diseases. Mol Psychiatry. 2021;26(10):5955-5966.

383. Tobler I, Gaus SE, Deboer T, et al. Altered circadian activity rhythms and sleep in mice devoid of prion protein. Nature. 1996;380:639-642.

384. Torres JM, Andreoletti O, Lacroux C, et al. Classical bovine spongiform encephalopathy by transmission of H-type prion in homologous prion protein context. Emerg Infect Dis. 2011;17(9):1636-1644.

385. Tycko R, Savtchenko R, Ostapchenko VG, Makarava N, Baskakov IV. The alpha-helical C-terminal domain of full-length recombinant PrP converts to an in-register parallel beta-sheet structure in PrP fibrils: evidence from solid state nuclear magnetic resonance. Biochemistry. 2010;49(44):9488-9497.

386. Tzaban S, Friedlander G, Schonberger O, et al. Protease-sensitive scrapie prion protein in aggregates of heterogeneous sizes. Biochemistry. 2002;41(42):12868-12875.

387. Urayama A, Morales R, Niehoff ML, Banks WA, Soto C. Initial fate of prions upon peripheral infection: half-life, distribution, clearance, and tissue uptake. FASEB J. 2011;25(8):2792-2803.

388. Urwin PJ, Mackenzie JM, Llewelyn CA, Will RG, Hewitt PE. Creutzfeldt-Jakob disease and blood transfusion: updated results of the UK transfusion medicine epidemiology review study. Vox Sang. 2016;110(4):310-316.

389. Uttley L, Carroll C, Wong R, Hilton DA, Stevenson M. Creutzfeldt-Jakob disease: a systematic review of global incidence, prevalence, infectivity, and incubation. Lancet Infect Dis. 2020;20(1):e2-e10.

390. Vallabh SM, Minikel EV. Implications of new genetic risk factors in prion disease. Nat Rev Neurol. 2021;17(1):5-6.

391. Vallabh SM, Minikel EV, Schreiber SL, Lander ES. Towards a treatment for genetic prion disease: trials and biomarkers. Lancet Neurol. 2020;19(4):361-368.

392. van Duijn CM, Delasnerie-Laupretre N, Masullo C, et al. Case-control study of risk factors of Creutzfeldt-Jakob disease in Europe during 1993-95. European Union (EU) Collaborative Study Group of Creutzfeldt-Jakob disease (CJD). Lancet. 1998;351(9109):1081-1085.

393. Vanni I, Pirisinu L, Acevedo-Morantes C, et al. Isolation of infectious, non-fibrillar and oligomeric prions from a genetic prion disease. Brain. 2020;143(5):1512-1524.

394. Vassileff N, Cheng L, Hill AF. Extracellular vesicles—propagators of neuropathology and sources of potential biomarkers and therapeutics for neurodegenerative diseases. J Cell Sci. 2020;133(23):jcs243139.

395. Vazquez-Fernandez E, Vos MR, Afanasyev P, et al. The structural architecture of an infectious mammalian prion using electron cryomicroscopy. PLoS Pathog. 2016;12(9):e1005835.

396. Vidal E, Fernandez-Borges N, Erana H, et al. Dogs are resistant to prion infection, due to the presence of aspartic or glutamic acid at position 163 of their prion protein. FASEB J. 2020;34(3):3969-3982.

397. Vitali P, Maccagnano E, Caverzasi E, et al. Diffusion-weighted MRI hyperintensity patterns differentiate CJD from other rapid dementias. Neurology. 2011;76(20):1711-1719.

399. Walker LC, Jucker M. Neurodegenerative diseases: expanding the prion concept. Annu Rev Neurosci. 2015;38:87-103.

400. Wang F, Wang X, Orru CD, et al. Self-propagating, protease-resistant, recombinant prion protein conformers with or without in vivo pathogenicity. PLoS Pathog. 2017;13(7):e1006491.

401. Wang F, Wang X, Yuan CG, Ma J. Generating a prion with bacterially expressed recombinant prion protein. Science. 2010;327(5969):1132-1135.

402. Wang LQ, Zhao K, Yuan HY, et al. Cryo-EM structure of an amyloid fibril formed by full-length human prion protein. Nat Struct Mol Biol. 2020;27(6):598-602.

403. Ward A, Hollister JR, Choi YP, et al. Altered distribution, aggregation, and protease resistance of cellular prion protein following intracranial inoculation. PLoS One. 2019;14(7):e0219457.

404. Watts JC, Giles K, Patel S, Oehler A, DeArmond SJ, Prusiner SB. Evidence that bank vole PrP is a universal acceptor for prions. PLoS Pathog. 2014;10(4):e1003990.

405. Watts JC, Westaway D. The prion protein family: diversity, rivalry, and dysfunction. Biochim Biophys Acta. 2007;1772(6):654-672.

406. Wegmann S, Miesbauer M, Winklhofer KF, Tatzelt J, Muller DJ. Observing fibrillar assemblies on scrapie-infected cells. Pflugers Arch. 2008;456(1):83-93.

407. Wells GAH, Scott AC, Johnson CT, et al. A novel progressive spongiform encephalopathy in cattle. Vet Rec. 1987;121:419-420.

408. Wickner RB. [URE3] as an altered URE2 protein: evidence for a prion analog in Saccharomyces cerevisiae. Science. 1994;264(5158):566-569.

409. Wickner RB. Yeast and fungal prions. Cold Spring Harb Perspect Biol. 2016;8(9):a023531.

410. Wickner RB, Edskes HK, Son M, Bezsonov EE, DeWilde M, Ducatez M. Yeast prions compared to functional prions and amyloids. J Mol Biol. 2018;430(20):3707-3719.

411. Wilham JM, Orrú CD, Bessen RA, et al. Rapid end-point quantitation of prion seeding activity with sensitivity comparable to bioassays. PLoS Pathog. 2010;6(12):e1001217.

412. Will RG, Ironside JW, Zeidler M, et al. A new variant of Creutzfeldt-Jakob disease in the UK. Lancet. 1996;347(9006):921-925.

413. Wille H, Govaerts C, Borovinskiy A, et al. Electron crystallography of the scrapie prion protein complexed with heavy metals. Arch Biochem Biophys. 2007;467(2):239-248.

414. Wille H, Michelitsch MD, Guenebaut V, et al. Structural studies of the scrapie prion protein by electron crystallography. Proc Natl Acad Sci U S A. 2002;99(6):3563-3568.

415. Williams A, Lucassen PJ, Ritchie D, Bruce M. PrP deposition, microglial activation, and neuronal apoptosis in murine scrapie. Exp Neurol. 1997;144(2):433-438.

416. Williams ES, Young S. Chronic wasting disease of captive mule deer: a spongiform encephalopathy. J Wildl Dis. 1980;16:89-98.

417. Wiseman FK, Cancellotti E, Piccardo P, et al. The glycosylation status of PrPC is a key factor in determining transmissible spongiform encephalopathy transmission between species. J Virol. 2015;89(9):4738-4747.

418. Wolf H, Grassmann A, Bester R, et al. Modulation of glycosaminoglycans affects PrPSc metabolism but does not block PrPSc uptake. J Virol. 2015;89(19):9853-9864.

419. Wolfe LL, Fox KA, Griffin KA, Miller MW. Mountain lions (Puma concolor) resist long-term dietary exposure to chronic wasting disease. J Wildl Dis. 2022;58(1):40-49.

420. Wong C, Xiong L-W, Horiuchi M, et al. Sulfated glycans and elevated temperature stimulate PrPSc dependent cell-free formation of protease-resistant prion protein. EMBO J. 2001;20:377-386.

421. Xu Y, Tian C, Wang SB, et al. Activation of the macroautophagic system in scrapie-infected experimental animals and human genetic prion diseases. Autophagy. 2012;8(11):1604-1620.

422. Yamasaki T, Suzuki A, Hasebe R, Horiuchi M. Retrograde transport by clathrin-coated vesicles is involved in intracellular transport of PrP(Sc) in persistently prion-infected cells. Sci Rep. 2018;8(1):12241.

423. Yim YI, Park BC, Yadavalli R, Zhao X, Eisenberg E, Greene LE. The multivesicular body is the major internal site of prion conversion. J Cell Sci. 2015;128(7):1434-1443.

424. Zanusso G, Nardelli E, Rosati A, et al. Simultaneous occurrence of spongiform encephalopathy in a man and his cat in Italy. Lancet. 1998;352(9134):1116-1117.

425. Zerr I, Kallenberg K, Summers DM, et al. Updated clinical diagnostic criteria for sporadic Creutzfeldt-Jakob disease. Brain. 2009;132(Pt 10):2659-2668.

426. Zerr I, Parchi P. Sporadic Creutzfeldt-Jakob disease. Handb Clin Neurol. 2018;153:155-174.

427. Zerr I, Schmitz M. Genetic prion disease. In: Adam MP, Ardinger HH, Pagon RA, et al., eds. GeneReviews((R)). University of Washington, Seattle. 1993.

428. Zhu C, Li B, Frontzek K, Liu Y, Aguzzi A. SARM1 deficiency up-regulates XAF1, promotes neuronal apoptosis, and accelerates prion disease. J Exp Med. 2019;216(4):743-756.

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