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Chapter 2

Virus Replication

Chapter Outline

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In the previous chapter, viruses were defined as obligate intracellular parasites that are unable to direct any independent biosynthetic processes outside the host cell. It was further noted that the genetic complexity of viruses varies greatly between individual virus families, ranging from those viruses that encode only a few proteins to others that encode several hundred proteins. Given this remarkable diversity, it is hardly surprising that the replication processes used by individual viruses would also be highly variable. However, all viruses must progress through the same general steps for replication to occur. Specifically, all viruses must attach to a susceptible host cell, enter the cell, disassemble the virus particle (uncoating), replicate its own genetic material and express the associated proteins, assemble new virus particles, and escape from the infected cell (release). This chapter will outline the general processes involved in each of these steps.

GROWTH OF VIRUSES

Before the development of in vitro cell culture techniques, viruses had to be propagated in their natural host. For bacterial viruses (bacteriophages), this was a relatively simple process. Consequently, scientists were able to develop laboratory-based research methods to study bacteriophages long before they were able to conduct comparable studies with plant or animal viruses. For animal viruses, samples from affected animals were collected and used to infect other animals, initially of the same species. When consistent results were obtained, attempts were usually made to determine whether other species might also be susceptible. These types of experiments were performed in an effort to determine the host range of any presumed viral agent. Although progress was made in defining the biological properties of viruses, this manner of propagation had obvious major drawbacks, especially with viruses affecting large animals. A most serious issue was the infection status of the recipient animals. For example, an undetected infectious agent in a sheep could alter the clinical signs observed after inoculation of that sheep with the test agent, and samples collected from this individual might now include several infectious agents, potentially confounding future experiments. In an attempt to avoid this type of contamination problem, animals that were to be used in research studies were raised under more defined conditions.

As new infectious agents were discovered and tests developed for their detection, the research animals became more “clean” and the concept of the “specific pathogen-free” (SPF) animal was born. It is noteworthy, however, that animals that were thought to be specific pathogen free could be infected with pathogens that were still undefined or undeclared. For example, pneumonia virus of mice (mouse pneumovirus) was discovered when “uninfected” control animals inoculated with lung extracts from other control animals died during experimental influenza virus infection studies. Many early virological and immunological studies were compromised by using rodents unknowingly infected with mouse hepatitis virus, lactate dehydrogenase-elevating virus, or other agents. Although live animals are no longer commonly used for routine virus isolation/propagation, animals are used still extensively for assessing viral properties such as virulence, pathogenesis, and immunogenicity.

The search for culture systems suitable for the propagation and study of viruses led to the discovery, in 1931,
that vaccinia virus and herpes simplex virus could be grown on the chorioallantoic membrane of embryonated chicken eggs, as was already known for fowlpox virus, a pathogen of birds. It was soon determined that viruses in many families of animal viruses can be grown in embryonated eggs, probably because of the wide variety of cell and tissue types present in the developing embryo and its environment. Consequently, the embryonated chicken egg became a standard culturing system for routine isolation and propagation of avian viruses and select mammalian viruses. In some cases, embryonated eggs entirely replaced research animals for the growth of virus stocks, and if the viral infection resulted in the death of the embryo, this system could also be used to quantify (titrate) the amount of virus in a virus stock or specimen (as described in greater detail later in this chapter). The egg system, which is labor-intensive and expensive, has largely been replaced by vertebrate cell culture-based systems; however, it is still widely used for the isolation and growth of influenza viruses and many avian viruses.

Various in vitro cell culture systems have been utilized since artificial medium was developed to maintain cell viability outside the source animal. These include organ cultures, explant cultures, primary cell cultures, and cell lines. An organ culture consists of an intact organ, which maintains the cellular diversity and the three-dimensional structure of the tissue. Organ cultures are utilized for short-term experiments. Explant cultures consist of portions (e.g., a slice or fragment) of an organ or tissue. Although explant cultures lack the complexity of the intact organ, their cellular components exist in a state that more closely models the in vivo environment than do cells propagated as primary cell cultures or cell lines. The creation of primary cell cultures utilizes proteases such as trypsin or collagenase to disassociate individual cells of a given tissue such as fetal kidney or lung. The individual cells are then permitted to attach to a cell culture matrix on which they will divide for a limited number of cell divisions. The limited lifespan of most primary cells requires continual production of the cells from new tissue sources, which can lead to variable cell quality between batches. This problem was largely overcome with the generation of immortalized cell lines, which in theory are capable of unlimited cell divisions. Initially, the generation of immortalized cell lines (transformation) was an empirical process with a low probability of success but it is now possible to immortalize virtually any cell type, so the number of cell lines representing different species is increasing rapidly.

The advent of in vitro animal cell culture brought research studies on animal viruses in line with those involving bacteriophage, and enhanced the quality and reliability of diagnostic testing. The ability to isolate and propagate animal viruses in cultured cells also made it possible to identify viruses as the etiologic agents of specific diseases through the successful application of Koch’s postulates. Fulfilment of Koch’s postulates requires that the infectious agent be isolated in pure culture; an achievement that was not possible for viruses prior to the development of cell culture systems. Replacement of living animals with cell culture systems decreased, but did not entirely eliminate, the problems associated with the presence of adventitious viruses. For example, early batches of the modified live poliovirus vaccine were contaminated with SV40 virus, a simian polyomavirus originating from the primary monkey kidney cultures used for vaccine production. Similarly, interpretation of the results of some early studies on newly described parainfluenza viruses is complicated because of virus contamination of the cell cultures used for virus isolation. Contamination of ruminant cell cultures with bovine viral diarrhea virus has been an especially insidious and widespread problem. Some contaminated cell cultures and lines were probably derived from infected fetal bovine tissue, but far more commonly, cells became infected through exposure to fetal bovine serum contaminated with bovine viral diarrhea virus. Fetal bovine serum became a standard supplement for cell culture medium in the early 1970s. The fact that many ruminant cell lines became infected from contaminated serum has compromised much research pertaining to ruminant virology and immunology, confounded diagnostic testing for bovine viral diarrhea virus and caused substantial economic losses as a result of contaminated vaccines. The extent of the problem was not fully defined until the late 1980s when high quality diagnostic reagents became available. As with experimental animals, problems with contaminating viral infections of cell cultures were only defined when the existence of the relevant infectious agent became known. Standard protocols for the use of serum in biological production systems now require irradiation of the serum to inactivate all viruses, known or unknown. With current technology allowing amplification and detection of virtually all nucleic acid species in cells, coupled with rapid sequencing of these products, a complete profile of cell cultures for contaminating organisms is now feasible.

**Recognition of Viral Growth in Culture**

Prior to the development of cell culture systems, identifying the presence of a viral agent in a plant or animal host was dependent upon the recognition of signs not found in an unaffected (control) host, death being the most extreme outcome and easiest to determine. Similarly, the presence of a replicating virus in cultured cells can be detected by identifying specific cellular characteristics that arise as a consequence of virus infection. In broad terms, any
observable cellular characteristic that is present in virus-infected cells and which is absent in uninfected cells maintained under identical growth conditions is referred to as a cytopathic effect (CPE). Virus-induced forms of CPE are generally observed through microscopic examination of the test culture system (Fig. 2.1). The most common forms of CPE observed in cultured cells are cell lysis and significant changes in cell morphology. Examples of morphological changes include the rounding, clumping, shrinkage, and detachment of individual cells from the cell culture matrix. Virus-induced fusion of neighboring cells represents another form of CPE. For example, cells infected with avian reovirus commonly fuse to form multinucleated cells or syncytia (Fig. 2.1A). Many members of the family Paramyxoviridae can cause this type of morphological change in cultured cells, but the extent of syncytium formation is cell type dependent. The type of cytopathology noted in culture can be characteristic for a given class of virus. For example, alphaherpesviruses produce distinct cytopathology characterized by rounded cells, with or without small syncytia, which spreads very rapidly through a susceptible cell culture (Fig. 2.1B).

Cells infected with some types of viruses acquire the ability to bind (adsorb) red blood cells (syn., erythrocytes) on their surface; a property referred to as hemadsorption. For example, cells infected with bovine parainfluenza virus 3 adsorb chicken red blood cells to the plasma membrane (Fig. 2.1D). Binding of red blood cells to the surface of the infected cell is actually mediated by viral glycoproteins that are expressed on the cell surface and which bind to receptors on the red blood cells. Consequently, hemadsorption only occurs with viruses that bud from the plasma membrane, and may be specific for red blood cells of a given animal species. Viruses that induce hemadsorption also show the ability to hemagglutinate red blood cells in cell-free medium. As discussed later in the chapter, this property can be used as the basis for quantifying the amount of virus within a sample. The same viral proteins that permit hemadsorption are also responsible for the hemagglutination reaction. There are, however, viruses that can themselves hemagglutinate red blood cells but not cause hemadsorption to cells infected with the same virus (eg, adenoviruses and alphaviruses).

Another type of morphological change commonly observed in virus-infected cells is the formation of inclusion bodies (Fig. 2.2). Inclusion bodies are intracellular abnormalities, commonly new structures, which arise as a direct consequence of virus infection. Inclusion bodies can be
observed with a light microscope after fixation and treatment with cytological stains, but, as with hemadsorption, not all viruses will produce obvious inclusion bodies. The type of virus infecting a cell can be inferred by the location and shape of the inclusions. For example, cells infected with herpesviruses, adenoviruses, and parvoviruses can have intranuclear inclusions, whereas cytoplasmic inclusions are characteristic of infections with poxviruses, orbiviruses, and paramyxoviruses (Fig. 2.2B,C). The composition of the inclusions will vary with the virus type. The cytoplasmic Negri bodies identified in rabies virus-infected cells are composed of aggregates of nucleocapsids, whereas the intranuclear inclusions that occur in adenovirus-infected cells consist of crystalline arrays of mature virus particles (Fig. 2.2D). Cytological stains are rarely used to identify cells infected with specific viruses, but are mainly used as a screening test to assess the presence of any virus.

In the absence of a metagenomic screening procedures, detection of viruses that produce no cytopathology (CPE), do not induce hemadsorption or hemagglutinate, or produce no definable inclusions, is accomplished using virus-specific tests. For example, this is the case in screening bovine cells for the presence of noncytopathic bovine viral diarrhea virus. The most commonly used tests in this type of situation are immunologically based assays such as the fluorescent antibody assay (immunofluorescence assay, IFA) or immunohistochemical staining assay (Fig. 2.3). The quality of these assays is dependent on the specificity of the antibodies that are used. With the development of monoclonal antibodies and monospecific antisera, this issue has been largely resolved. Other virus-specific tests are based on the detection of virus-specific nucleic acid in the infected cells. Initially, assays of this sort relied on the use of nucleic acid probes capable of hybridizing in a sequence-specific manner with the target nucleic acid. Hybridization-based assays have largely been replaced by those based on polymerase chain reaction (PCR) because of their enhanced sensitivity and ease of performance (see Chapter 5: Laboratory Diagnosis of Viral Infections).
VIRUS REPLICATION

A fundamental characteristic that separates viruses from other replicating entities is the manner in which new virus particles are synthesized. Unlike eukaryotic and prokaryotic cells, which increase their numbers through the processes of mitosis and binary fission, respectively, new virus particles are assembled de novo from the various structural components that are synthesized during the virus infection. The earliest recognition of this unique replication pattern came from studies using bacteriophage. The outline of the experimental proof of concept was relatively simple: (1) add a chloroform-resistant phage to a culture of bacteria for several minutes; (2) rinse the bacteria to remove nonattached phage; (3) incubate the culture and remove samples at various periods of time; (4) treat sampled bacterial cultures with chloroform to stop growth; (5) quantify the amount of phage at each of the time periods. The result of this type of experiment is what we now refer to as a one-step growth curve, which in principle, can be performed with any virus that can be propagated in cell culture (Fig. 2.4). The remarkable finding of this type of study was that infectious virus “disappeared” from the infected cultures for a variable period of time, depending on the virus—host-cell system. This is referred to as the eclipse period, and represents the period of time that begins with cell entry/uncoating and ends with the appearance of newly formed infectious virus particles. Following the end of the eclipse period there is an essentially exponential increase in production of infectious virus particles until the host cell is unable to maintain its metabolic integrity. Depending on the type of virus, there may be sudden release of virus particles following lysis of the host cell, as exemplified by T-even bacteriophages, or a prolonged release of virus particles via sustained budding of virus particles at a cell membrane site, such as with influenza A virus.

The one-step growth curve can be used to divide the virus replication cycle into its component parts, which include attachment, the eclipse period (entry, uncoating, replication of component parts, virion assembly), and release of virus particles. Although the replication cycles of all conventional viruses follow these same general

FIGURE 2.3 Indirect fluorescent antibody detection of noncytopathic bovine viral diarrhea virus (BVDV)-infected cells. Bovine cells were exposed to BVDV for 72 hours and then fixed with cold acetone. Fixed cells were probed with a BVDV-specific monoclonal antibody (20.10.6) followed by staining with a fluorescein isothiocyanate (FITC)-conjugated polyclonal goat anti-mouse serum. Courtesy of E. Dubovi, Cornell University.

FIGURE 2.4 One-step growth curve of a nonenveloped virus. Attachment and penetration are followed by an eclipse period of 2–12 hours during which cell-associated infectivity cannot be detected. This is followed by a period of several hours during which virus maturation occurs. Virions of nonenveloped viruses are often released late and incompletely when the cell lyses. The release of most enveloped virions occurs concurrently with maturation by budding from the plasma membrane.
steps, the details of each step can vary widely depending on the specific virus. Therefore, the kinetics of the one-step growth curve differs with the unique properties of the specific virus—host-cell system used. To ensure that all steps of the virus replication cycle are temporally synchronized, it is important that the infection be initiated with enough virus particles to simultaneously infect all cells in the culture. This is achieved by using a high multiplicity of infection [typically 10 plaque forming units (pfu) of virus/cell].

A discussion focusing on the individual steps of the general virus replication cycle now follows. This discussion includes expanded descriptions and details of complete replication cycles for model viruses representing four major groups [positive strand RNA viruses (picornavirus), negative strand RNA viruses (rhabdovirus), retroviruses, and DNA viruses (adenovirus)]. More comprehensive discussions covering the specific details of individual virus families are found in Part II of this book.

**Attachment**

The critical first step in the virus replication cycle is the attachment of the virus particle to a host cell. Attachment requires specific interactions between components of the virus particle (eg, capsid proteins or envelope glycoproteins) and components of the host cell (eg, a glycoprotein or carbohydrate moiety). This process can be conceptually simple whereby attachment can involve interactions between a single component of the virus with a single component of the cell. For example, binding of influenza virus to a host cell requires only an interaction between the viral hemagglutinin (HA) glycoprotein and a sialic acid residue on the cell surface. Alternatively, attachment-related interactions can be complex and involve sequential interactions between multiple components of both the virus and the cell. Examples of this type of cell binding are described below in the expanded discussions of the adenovirus and retrovirus replication cycles. Many host proteins are not widely expressed but instead are expressed in a cell- or tissue-specific manner. Therefore, receptor usage plays an important role in defining the tissue/organ specificity (tropism) of a virus. In turn, the tissue and organ specificity of a virus largely defines its pathogenic potential and the nature of the disease it causes. Similarly, cellular components (eg, proteins, carbohydrate structures, etc.) can differ markedly between organisms, thus, receptor usage also influences the types of organisms (host species) that a virus can infect (host range).

Virus particles interact with cell-surface molecules which are referred to as receptors, coreceptors, attachment factors, or entry factors depending on the role(s) that they play in the attachment and entry processes. Frequently, the term “viral receptor” is used to describe these cell-surface molecules, which is something of a misnomer, as cells certainly do not maintain receptors for the purpose of binding viruses. Rather, viruses have evolved to use host cell molecules that perform functions related to normal cellular processes. Initial contact of a virus particle with the cell surface often involves short-distance electrostatic interactions with charged molecules such as heparan sulfate proteoglycans. This initial contact may simply help to concentrate virus on the surface of the cell, which facilitates the establishment of more specific interactions with other receptor-like molecules. The affinity of binding between an individual virus component and its cellular ligand may be low; however, the virus surface possesses many receptor binding sites, thus the affinity of binding between the virus and the host cell is enhanced by the establishment of multiple virus/receptor interactions. Although viruses require at least one receptor to be expressed on the surface of the host cell, some viruses must also engage an intracellular receptor(s) in order to initiate a productive infection. These intracellular interactions do not play a role in attachment to the cell but instead are required for the final stages of the entry/uncoating process; and therefore, will be discussed in more detail below.

The identification of host cell factor(s) that serve as receptors for virus attachment is important for understanding the molecular details of specific virus replication cycles, and also has practical implications as this knowledge can inform the design of antiviral drugs. In recent years, numerous host cell components capable of functioning as receptors-entry factors for viruses have been identified. These include ligand-binding receptors (eg, chemokine receptors, transferrin receptor 1), signaling molecules (eg, CD4), cell adhesion/signaling receptors (eg, intercellular adhesion molecule-1, ICAM-1), enzymes, integrins, and glycoconjugates with various carbohydrate linkages, sialic acid being a common terminal residue (Table 2.1). As shown in Table 2.1, different viruses may use the same receptor/entry factor (eg, Coxsackievirus and some adenoviruses), which results in these viruses having a shared or overlapping cell/tissue tropism. The number and identity of host cell molecules that play a part in the initial interactions of virus with host cells will certainly increase as new viruses are identified and as existing viruses are better characterized.

The process of identifying receptors/entry factors is more complicated than initially imagined, as viruses within a given family may use different receptors. Furthermore, different strains of the same virus can utilize different receptors and adaptation of a virus to growth in cell culture can change receptor usage of the virus. For example, wild-type strains of foot and mouth disease virus bind to integrins in vivo, but cell culture-passaged strains
| Virus                                      | Family         | Receptor                                                                 |
|--------------------------------------------|----------------|--------------------------------------------------------------------------|
| Human immunodeficiency virus               | Retroviridae   | CCR5, CCR3, CXCR4 (heparan sulfate proteoglycan)                         |
| Avian leukemia/sarcoma virus               | Retroviridae   | Tissue necrosis factor-related protein TVB                               |
| Murine leukemia virus E                     | Retroviridae   | MCAT-1                                                                   |
| Bovine leukemia virus                      | Retroviridae   | BLV receptor 1                                                           |
| Poliovirus                                 | Picornaviridae | PVR (CD155)—Ig family                                                  |
| Coxsackieviruses B                         | Picornaviridae | Coxsackievirus and adenovirus receptor (CAR)—Ig family                  |
| Human rhinovirus 14                        | Picornaviridae | Intercellular adhesion molecule-1 d(ICAM-1)—Ig family                   |
| Echovirus 1                                | Picornaviridae | αβ integrin VLA-2                                                        |
| Foot and mouth disease virus—wild-type virus |                | Various integrins                                                        |
| Foot and mouth disease virus—cell-culture-adapted |     | Heparan sulfate proteoglycan                                              |
| Feline calicivirus                         | Caliciviridae  | Feline junction adhesion molecule-A (fJAM-A)                            |
| Adenovirus 2                               | Adenoviridae   | CAR-Ig family                                                            |
| Adenoviruses                               | Adenoviridae   | αβ integrins, αβ integrins                                               |
| Herpes simplex virus 1                     | Herpesviridae  | Herpes virus entry mediator A (HveA), heparan sulfate proteoglycan, others |
| Human cytomegalovirus                      | Herpesviridae  | Heparan sulfate proteoglycan                                              |
| Epstein—Barr virus                         | Herpesviridae  | CD21, complement receptor 2 (CR2)                                        |
| Pseudorabies virus                         | Herpesviridae  | CD155—Ig family                                                          |
| Feline parvovirus                          | Paroviridae    | Transferrin receptor-1 (TfR-1)                                           |
| Adeno-associated virus 5                   | Paroviridae    | α(2,3)-linked sialic acid                                                |
| Influenza A virus                          | Orthomyxovirida| Sialic acid                                                              |
| Influenza C virus                          | Orthomyxovirida| 9-O-acetylsialic acid                                                    |
| Canine distemper virus                     | Paramyxovirida | Signaling lymphocyte activation molecule (SLAM); Nectin 4               |
| Newcastle disease virus                    | Paramyxovirida | Sialic acid                                                              |
| Bovine respiratory syncytial virus         | Paramyxovirida | Unknown                                                                  |
| Hendra virus                               | Paramyxovirida | Ephrin-B2                                                                |
| Rotavirus                                  | Reoviridae     | Various integrins                                                        |
| Reovirus                                   | Reoviridae     | Junction adhesion molecules (JAMs)                                       |
| Mouse hepatitis virus                      | Coronaviridae  | Carcinoembryonic antigen (CEA)—Ig family                                |
| Transmissible gastroenteritis virus        | Coronaviridae  | Aminopeptidase N                                                         |
| Lymphocytic choriomeningitis virus         | Arenaviridae   | α-Dystroglycan                                                            |
| Dengue virus                               | Flaviviridae   | Heparan sulfate proteoglycan                                              |
| Rabies virus                               | Rhabdoviridae  | Nicotinic acetylcholine receptor (nAchR), Neuronal cell adhesion molecule (NCAM) |
of the virus can use heparan sulfate. This change in receptor specificity alters the pathogenicity of the virus, clearly indicating that receptor usage influences the disease process. Some viruses with a broad host range, such as arthropod-borne viruses and some of the alphaherpesviruses, are thought to use several different host-specific receptors, which accounts for their ability to grow in cells from many hosts. Alternatively, a virus can use a common receptor that is expressed in multiple host species. For example, Sindbis virus was recently shown to utilize a protein called natural resistance-associated macrophage protein (NRAMP) as a receptor in insect cells, and to use the mammalian homolog (NRAMP2) for binding to cultured mammalian cells and in the tissues of mice.

Two additional issues related to the virus/cell attachment process are notable. A model was recently proposed in which cell receptors that normally function in the recognition and clearance of apoptotic cells are used for cell attachment/entry by dengue virus and perhaps by related flaviviruses. Flaviviruses bud through the membrane of the endoplasmic reticulum, and consequently, are thought to incorporate phosphatidylserine (PtdSer) into the outer leaflet of the viral envelope. PtdSer is also enriched on the outer leaflet of the plasma membrane of cells undergoing apoptosis due to lipid reshuffling, and is bound directly or indirectly by members of the TIM and TAM families of transmembrane receptor proteins, respectively. TIM and TAM proteins are expressed by a number of cell types including macrophages and dendritic cells, which are normal targets of dengue virus infection. Under normal circumstances the binding between the TIM/TAM proteins on myeloid cells and the PtdSer on apoptotic cells leads to the uptake and clearance of the apoptotic cell. By incorporating PtdSer into the viral envelope, dengue virus is thought to mimic an apoptotic cell, enabling the virus to be bound and internalized by cells expressing the TIM/TAM proteins. This mechanism does not appear to be unique to flaviviruses as a similar model has been proposed for vaccinia virus (family Poxviridae). A second somewhat indirect mechanism of cell binding/entry is also best exemplified by dengue virus. This mechanism is referred to as antibody-dependent enhancement of infection, which occurs when the virus particle is bound by nonneutralizing IgG antibodies that in turn are bound by activating Fcγ receptors expressed on the surface of mononuclear phagocytic cells (eg, macrophages). This interaction leads to internalization of the antibody/virus complex and eventual release of infectious virus into the cytoplasm of the phagocytic cell. Foot and mouth disease virus and feline coronavirus can also infect cells through this antibody-mediated enhancement mechanism in vitro, but its importance in the natural infection process is conjectural.

**Entry and Uncoating**

The binding of a virus to a receptor on a host cell represents the first step in the replication cycle; however it will not result in a productive infection unless this event leads to entry of the virus into the cell with subsequent uncoating of the virus particle and release the viral genome into the proper intracellular compartment (cytoplasm or nucleus depending on the virus). Though the plasma membrane is only about 7 nm thick it serves as an effective physical barrier that blocks the free passage of viruses into the cell. However, viruses have evolved a range of strategies for breaching this barrier and gaining access to the cell interior. Depending on the specific virus, uncoating of the virus particle occurs after the particle has entered the cell or concurrently with the cell entry process. The virus particle is metastable, which means that its structure is generally stable enough to move as a physical entity from cell to cell or from one host to another, but it is primed to undergo structural rearrangements and to disassemble when exposed to the proper biological stimuli. As detailed below, the biological stimuli that induce the entry and/or uncoating processes for different viruses include, but are not limited to, binding to specific host cell proteins, proteolysis by host cell enzymes, and exposure to acidic pH. This section of the chapter will focus on general mechanisms of virus entry into host cells, and will include multiple examples of specific virus/host interactions that initiate the uncoating process.

A bound virus particle (or sometimes the virus genome alone) enters a host cell by one of two general mechanisms: (1) direct entry across the plasma membrane or (2) entry into the cell within a membrane-bound vesicle. As described below, viruses that enter the cell within membrane-bound vesicles must still pass through a limiting membrane to gain access to the cytosol. In both of these mechanisms the receptor molecule(s) assists in the entry process, and the nature of the receptor can determine the mechanism by which the virus enters the host cell. Mechanisms of direct entry across the plasma membrane will be addressed first. Cell entry by the picornaviruses will be used as an example of direct entry by a nonenveloped virus, and the mechanism presented is based on the current model for direct entry of poliovirus. Poliovirus attaches to a host cell by binding to the poliovirus receptor (PVR, CD155). Noncovalent interactions established between the poliovirus receptor and proteins that form the capsid induce significant changes in the capsid structure. Most significantly, a protein located inside the capsid (VP4) is expelled from the virus particle, and capsid protein VP1 undergoes conformational changes that cause the hydrophobic N-terminus of the protein to
translocate from the capsid interior to the capsid surface where it is inserted into the plasma membrane of the host cell. The N-terminal sequences of multiple VP1 proteins are thought to associate and form a pore in the plasma membrane through which the genomic RNA is released into the cytoplasm of the host cell (Fig. 2.5).

All enveloped viruses must mediate the process of membrane fusion to enter their host cell. For enveloped viruses that achieve direct entry at the cell surface, fusion occurs between the virus envelope and the plasma membrane, and this process occurs under neutral pH conditions (pH-independent entry). This mode of cell entry is characteristic of paramyxoviruses (eg, Newcastle disease virus and measles virus) and some (eg, human immunodeficiency virus, HIV) but not all retroviruses. The initial stages of the entry process for these viruses are conceptually similar to those of poliovirus in that binding of the virus to an appropriate receptor molecule stimulates conformational changes in a viral protein that in turn facilitates passage of the viral genome into the cell. In this case, conformational changes occur within a spike-associated glycoprotein that transitions from a native conformation (prefusion conformation) into an alternate conformation (postfusion conformation) that is capable of mediating fusion between the viral envelope and the plasma membrane. In the case of Newcastle disease virus, binding of host cell receptors is performed by the hemagglutinin neuraminidase (HN) glycoproteins, which form homotetrameric spikes that project from the virion surface (Fig. 2.6). Receptor binding stimulates conformational changes in HN, which in turn destabilize and induce conformational changes in a neighboring protein called the fusion (F) protein. F proteins consist of disulfide-like heterodimers (F1/F2), which assemble into homotrimeric spikes. The N-terminal sequences of F1 are highly hydrophobic and are referred to as a “fusion peptide.” Prior to receptor binding, the F proteins assume their prefusion conformation, in which the fusion peptide sequences are sequestered from the hydrophilic environment that surrounds the virus. Following receptor binding by HN, conformational changes are induced in the F proteins that result in the projection of the fusion peptides towards the host cell where they insert themselves into the lipid bilayer of the plasma membrane. Continued conformational changes in the F proteins draw the plasma membrane towards the virus envelope. When the two membranes make contact, mixing of their lipids occurs and eventually the membranes are fused together to form a fusion pore. As the fusion pore grows in size the viral envelope becomes fully incorporated into the plasma membrane of the cell and the genome of the virus is released into the cytoplasm of the host cell. In both of the virus systems just described (poliovirus and Newcastle disease virus), the entry and uncoating processes occur simultaneously and are only initiated after the virus particle has bound to a biologically-relevant receptor protein.

Natural receptor/ligand interactions at the cell surface often initiate signaling pathways and cellular processes that lead to the internalization of the receptor/ligand complex into a membrane-bound vesicle. Many viruses have evolved strategies to exploit these same signaling pathways and cellular processes to gain entry into the host cell. Endocytosis is the general mechanism whereby extracellular materials are internalized in membrane-bound vesicles (Fig. 2.7). One form of endocytosis is
termed clathrin-mediated endocytosis. Briefly, clathrin-mediated endocytosis begins with the diffusion of receptor/ligand complexes to invaginations in the membrane that are coated on their cytoplasmic side by a polymeric lattice composed of the clathrin protein. These conformational changes result in the insertion of the fusion protein components of F into the plasma membrane of the host cell. Continued conformational changes in F draw the two membranes together, facilitate the mixing of lipid components, and lead to the formation of the fusion pore. From Smith, E.C., Popa, A., Chang, A., Masante, C., Dutch, R.E., 2009. Viral entry mechanisms: the increasing diversity of paramyxovirus entry. FEBS J. 276, 7217–7227. Reprinted by permission of John Wiley & Sons, Inc.

FIGURE 2.6 Membrane fusion mediated by the trimeric fusion (F) glycoprotein spike of Newcastle disease virus. A. Sequential stages of membrane fusion and fusion pore formation involving the envelope of Newcastle disease virus and the plasma membrane of a host cell. B. The prefusion conformation of the F glycoprotein spike is altered in response to binding of a host cell receptor by the hemagglutinin–neuraminidase (HN) spike. These conformational changes result in the insertion of the fusion protein components of F into the plasma membrane of the host cell. Continued conformational changes in F draw the two membranes together, facilitate the mixing of lipid components, and lead to the formation of the fusion pore.

FIGURE 2.7 Endocytic mechanisms of cell entry. Endocytosis in animal cells can occur via several different mechanisms. Several mechanisms are defined as pinocytic—that is, they involve the uptake of fluid, solutes, and small particles. These include clathrin-mediated, macropinocytosis, caveolar/raft-mediated mechanisms, in addition to several novel mechanisms. Some of these pathways involve dynamin-2, as indicated by the beads around the neck of the endocytic indentations. Large particles are taken up by phagocytosis, a process restricted to a few cell types. In addition, there are pathways such as IL-2, the so-called GEEC pathway, and the flotillin- and ADP-ribosylation factor 6 (Arf6)-dependent pathways that carry specific cellular cargo but are not yet used by viruses. Adeno 2/5, Adeno 3, adenoviruses 2/5 and 3; CME, clathrin-mediated endocytosis; HPV-16, human papillomavirus 16; HSV-1, herpes simplex virus 1; LCMV, lymphocytic choriomeningitis virus; mPy, mouse polyomavirus; SFV, Semliki Forest virus; SV40, simian virus 40; VSV, vesicular stomatitis virus. From Mercer, J., 2010. Virus entry by endocytosis. Annu. Rev. Biochem. 79, 6.1–6.31. Copyright © 2010 by Annual Reviews, with permission.
the early endosomal pathway. Endosome contents will subsequently be delivered to late endosomes and eventually to endolysosomes. As the endosome vesicles progress through the pathway their interior pH becomes increasingly acidic and the composition of resident cell proteins changes. For some viruses, the acidic pH within the endosome serves as the stimulus for structural changes in the virus particle that facilitate exit from the endosome and uncoating of the virion (pH-dependent entry). This process has been studied in detail using influenza A virus. Attachment of influenza A virus to a host cell is mediated by the viral hemagglutinin (HA) spike, a homotrimeric structure composed of three disulfide-linked HA1/HA2 heterodimers. The HA spike binds to sialic acid residues on the cell surface and bound virus particles are then taken into the cell within endosomes. The decreasing pH within the endosome induces profound conformational changes in HA that cause the N-terminal sequences of HA2, which function as a fusion peptide, to extend outward from the virion and insert into the endosome membrane. Much like the F proteins of Newcastle disease virus, the HA proteins continue to refold, drawing the virus envelope and the endosome membrane together and eventually causing them to fuse. Fusion between the two membranes results in the release of the virus genome into the cytoplasm. The genome of influenza A virus consists of eight nucleocapsids (negative sense RNAs complexed throughout their length by NP protein) that are associated with each other and with multiple copies of the M1 protein. The M1 protein appears to aggregate the eight nucleocapsids through noncovalent protein:protein interactions. As the endosome becomes acidified, hydrogen ions are transported through a virion-associated ion channel (M2 ion channel) to acidify the virion interior. The drop in intraparticle pH dissociates M1 from the complex which allows the nucleocapsid aggregate to disassemble into individual nucleocapsids that are small enough to be imported into the nucleus through a nuclear pore. Thus, in the case of influenza A virus, exposure to acidic pH stimulates two separate uncoating processes (ie, membrane fusion and release of individual nucleocapsids). As predicted, infection of cells by those viruses that enter via clathrin-mediated endocytosis is inhibited by compounds that prevent endosome acidification (eg, bafilomycin A1, chloroquine, NH₄Cl). Some viruses that enter the cell through clathrin-mediated endocytosis require biological stimuli beyond exposure to acidic pH in order to escape from the endosome compartment. Ebola virus mediates membrane fusion at the late endosome or endosome/lysosome stage of the pathway. Membrane fusion is mediated by the GP spike glycoprotein that consists of GP1/GP2 heterodimers. The GP protein does not become fully primed for membrane fusion until it is cleaved by two host cell proteases (cathepsin L and cathepsin B), which the virus does not encounter until it reaches the late endosome/lysosome. In addition, primed GP is not stimulated to perform the conformational changes required for membrane fusion until it has bound to an internal receptor called Niemann-Pick C1 (NPC1), which is a resident protein of the late endosome—lysosome membrane. The ability of Lassa virus (family Arenaviridae) to exit the endosome also depends on binding to an internal receptor.

A second major endocytosis pathway that is exploited by viruses for entry into host cells is the caveosome system (Fig. 2.7). In this pathway, viruses bound at the cell surface enter small membrane invaginations called caveolae. Caveolae are coated on their cytoplasmic side by caveolin proteins. Similar to the endosome system, the invaginations can be bound to cargo molecules and pinch off the plasma membrane to form vesicles called caveosomes. Unlike the endosomal system, the caveosomes maintain a neutral pH within the vesicle. However, there appears to be a pathway for caveosomes to enter the endosomal system, which would allow pH activation of some viruses. Alternatively, caveosomes can be delivered to the endoplasmic reticulum. Virus entry through the caveosome system has been studied extensively using SV40 virus (family Polyomaviridae). As a general rule, enveloped viruses do not use the caveosome system; this may be a function of particle size, as the vesicles formed by the endosomal system are larger and can accommodate the generally larger size of virions that possess lipid envelopes.

In many cases, the entry of the virus particle, nucleocapsid, or genomic nucleic acid into the cytoplasm is not the final step in the initiation of the replication process for the virus. Commonly, the initial steps of the virus entry and uncoating processes do not result in release of the genome in a form that can initiate replication-related processes (eg, translation, transcription, or replication). Furthermore, these early events often do not place the viral genome in the proper cellular compartment for replication. Again, cellular processes are involved in stimulating additional uncoating processes, and in the transport of the viral units to the required location. For example, Semliki forest virus (family Togaviridae) enters the cell by clathrin-mediated endocytosis and fusion of the virus envelope with the endosome membrane releases theicosahedral nucleocapsid into the cytoplasm. The capsid structure is then disassembled following binding of the capsid proteins by the cell’s 60S ribosomal subunits. Disassembly of the capsid releases the viral positive strand RNA, which is then available to be translated into the viral proteins that will orchestrate downstream replication processes. Similarly, the initial steps of cell entry by adenovirus (described in more detail below) deliver a modified virus particle to the cytoplasm, but replication of the adenovirus DNA occurs in the nucleus. Therefore, translocation of viral components from the cytoplasm to the nucleus is a required step in infection by almost all DNA viruses (poxviruses are a notable exception). For most of the longer
translocation needs, the microtubule transport system is used, and movement of the virus particle is often facilitated by molecular motors such as dynein or kinesin. Actin filaments can also be utilized for more localized movements. For the DNA viruses and RNA viruses such as influenza virus that utilize the nucleus for their replication site, nuclear localization signals exist on key viral proteins that interact with soluble cellular proteins of the nuclear import system. These proteins link the viral units to the nuclear pore complex, either permitting translocation of the viral unit into the nucleus (paroviruses) or inducing the transport of the nucleic acid into the nucleus (adenoviruses, herpesviruses). The replication cycle of individual virus families are described in more detail in Part II of this book.

Viral Protein and Nucleic Acid Synthesis

Up to this point in the replication process, the virus particle has been somewhat passive as no biosynthetic activity directed by the viral genome has occurred. The preliminary steps of the infection process have placed the viral genome in position to take active control of the replication cycle and to remodel the cell to assist in the production of progeny virus particles. The details of the next phases of the replication cycle, which include the expression of viral proteins and replication of the viral genome, differ markedly between viruses, and play a major role in determining the evolutionary relationships between viruses and in the placement of viruses into proper taxonomic groupings. Examples of four different replication strategies will be described in succeeding pages in order to emphasize specific aspects of virus replication and to demonstrate the diversity of replication strategies.

Representative Examples of Virus Replication Strategies

Picornaviruses

The family Picornaviridae includes a number of important pathogens of animals and humans, for example poliovirus, hepatitis A virus, and foot and mouth disease virus (see Chapter 26: Picornaviridae). Picornaviruses are small, relatively simple, nonenveloped viruses. The virus particle has an icosahedral symmetry and consists of a protein capsid and a genome comprised of a single strand of positive sense RNA. The genomic RNA contains a small virus-encoded protein (VPg) covalently bound to its 5’ end and a genetically encoded 3’ poly A tail. The picornavirus entry process differs depending on the specific virus. The capsid of some picornaviruses (eg, poliovirus) undergoes conformational changes at the plasma membrane in response to receptor binding, and these changes are thought to create a transmembrane protein pore through which the virus genome is extruded from the virion into the cytoplasm of the host cell (as described above, Fig. 2.5). Other picornaviruses, such as foot and mouth disease virus, enter cells via receptor-mediated endocytosis and release their genome into the cytoplasm following conformational changes induced by the acidification of the endosome. Regardless of the mechanism used, the entry process results in release of the genomic RNA into the cytoplasm of the host cell where it will be used as a template for protein synthesis and for the replication of new viral genomes as depicted in Fig. 2.8.

Shortly after the genomic RNA is released into the cytoplasm, the VPg protein is removed from the RNA by a cellular enzyme that normally functions in the repair of cellular DNA. Following the removal of VPg, the RNA associates with the cellular translational system and is used as a template for synthesis of the viral proteins. However, unlike most host-cell mRNAs, the picornavirus genomic RNA lacks a standard 5’ cap structure which is normally required to initiate the assembly of a ribosome onto the mRNA template (cap-dependent translation). Therefore, picornaviruses have had to evolve a mechanism for assembling host cell ribosomes onto viral mRNAs in the absence of a 5’ cap (cap-independent translation). This function is provided by RNA sequences located near the 5’ end of the genomic RNA itself. In picornaviruses, the AUG codon that is used to initiate translation is located an unusually long distance from the 5’ end of the RNA (743 nt in the case of poliovirus). The long nontranslated region between the 5’ end and the AUG start site assumes multiple secondary and tertiary structures due to extensive intramolecular base pairing. The majority of this region is referred to as the internal ribosome entry site (IRES) based on its ability to interact with cellular components of the translational machinery and to assemble ribosomes internally on the RNA a short distance upstream of the start codon. As virus replication proceeds, translation of cellular proteins decreases markedly as ribosomes are assembled almost exclusively onto viral mRNAs. The restriction of cellular protein synthesis is due to the cleavage and subsequent inactivation of the translation initiation factor eIF4G by a virus-encoded protease (designated L protease or 2A protease depending on the virus). eIF4G is required for cap-dependent translation and in its absence ribosomes are not assembled on capped mRNA. Translation of viral mRNA is not affected by cleavage of eIF4G as these mRNAs lack a cap and ribosomes are assembled internally on viral mRNAs by the IRES. The selective inhibition of cellular translation reduces competition for ribosomes and reduces the ability of the cells to produce an array of antiviral molecules such as type I interferons that are made in response to the viral infection (see Chapter 4: Antiviral Immunity and Virus Vaccines).
The genomic RNA of picornaviruses includes only a single open reading frame that is translated into a single large polyprotein that is subsequently cleaved by virus-encoded proteases (which are embedded within the polyprotein) into the individual structural and nonstructural proteins of the virus. Intermediate cleavage products are designated P1, P2, and P3 (Fig. 2.8). Proteins that are used to assemble the capsid (VP1, VP2, VP3, and VP4) are ultimately derived from P1. Proteins required for genome replication and interference with host cell processes are ultimately derived from P2 and P3. The input genomic RNA will be translated repeatedly to generate virus proteins but eventually it will be used as a template for replication. Replication of the picornavirus RNA is performed in close association with remodeled cellular membranes and requires most of the proteins derived from P2 and P3.
from the P2 and P3 precursor proteins as well as several cellular proteins. The host cell does not provide an RNA-dependent RNA polymerase (RdRp) enzyme capable of replicating the viral RNA genome; and therefore, picornaviruses (and nearly all other RNA viruses) have evolved their own RdRp enzyme for this purpose. Picornaviruses encode an RdRp enzyme called 3D\text{pol}, which is derived from the P3 precursor protein. 3D\text{pol} is a primer-dependent polymerase and the primer that is used in the replication process is the VPg protein itself. A tyrosine residue within VPg donates the hydroxyl group onto which two uridine nucleosides are added by 3D\text{pol} to form VPg–U–U–OH. The addition of the uridine nucleosides is templated by two adenosine nucleosides located in the non-base paired region of a RNA stem loop structure located internally on the genomic RNA. This stem loop structure is referred to as the cis-acting replication element (CRE). The actual sequence and internal location of CRE varies among different picornaviruses but all contain two or more adjacent adenosine residues within their loop structure which serve as the template for the uridylation of VPg. Following its synthesis, the VPg–U–U–OH primer is translocated to the terminal sequences of the 3’ poly A tract where it is hybridized to the RNA through A:U base pairing. The VPg–U–U–OH primer is then extended by 3D\text{pol} to form a full-length complementary negative strand. The negative strand terminates in at least two adenosine residues, which facilitates base pairing with another VPg–U–U–OH primer and the synthesis of positive strand RNAs. Many of the newly synthesized positive strand RNAs will be used as mRNAs following the enzymatic removal of VPg. Other positive strand RNAs will retain VPg and be packaged into progeny virions.

The capsid structure of picornaviruses consists of multiple copies of a structural subunit called the protomer. The protomer of most picornaviruses contains single copies of the structural proteins VP1, VP3, VP0 (a precursor to VP2 and VP4), each of which is derived from P1. Sixty protomers associate through noncovalent interactions to form the icosahedral capsid. The exact mechanism by which the RNA genome is incorporated into the developing capsid remains unclear, but two primary models have been proposed. The first model proposes that individual protomers assemble on a genomic RNA and incorporation of the genome occurs coincident with the capsid assembly process. The second model proposes that protomers interact in the absence of RNA to form empty capsid structures into which the genomic RNA is then somehow inserted. In both models, the final step of capsid maturation involves cleavage of VP0 into VP2 and VP4 by what is believed to be an autoproteolytic process. The rate-limiting process for particle maturation appears to be the availability of VPg-containing RNA. All steps of picornavirus virion assembly occur intracellularly, and late in infection crystalline arrays of virus particles form in the cytoplasm of infected cells. Ultimately, these virus particles are released from the cell en mass following dissolution of the cell structure.

The replication cycle of picornaviruses illustrates several properties that are common to many positive strand RNA-based viruses. First, the RNA genome is infectious, meaning that the genomic RNA itself is capable of initiating a productive infection when introduced into a host cell in the absence of any viral proteins. Second, the positive sense genomic RNA is able to associate with ribosomes and serve as a template for the production of viral proteins which then perform the processes of replicating the viral RNA and of manipulating critical host-cell metabolic and defense-related processes. Third, viral proteins can be synthesized as larger precursor proteins (polyproteins) that are subsequently resolved into the individual structural and nonstructural proteins by virus-encoded proteases. Finally, these viruses often induce the remodeling of cellular membrane structures that provide sites for viral RNA synthesis.

**Rhabdoviruses**

Vesicular stomatitis virus is the prototypical member of the *Rhabdoviridae* family (see Chapter 18: *Rhabdoviridae*), and the following description of rhabdovirus replication is based on the replication cycle of this virus (Fig. 2.9). Rhabdoviruses are enveloped viruses that have a distinctive bullet-shaped morphology. The rhabdovirus genome consists of a single strand of negative sense RNA. Unlike the genomic RNA of picornaviruses, the genomic RNA of rhabdoviruses is not naked, but instead exists as a nucleocapsid consisting of an RNA complexed throughout its length with repeating copies of the nucleocapsid (N) protein (1 N protein:9 nt of RNA). Infection of a host cell is initiated by attachment of the virus glycoproteins (G) to receptors expressed on the plasma membrane, and cell entry via receptor-mediated endocytosis. Decreasing pH within the endosomal vesicle induces conformational changes in the G proteins, which in turn mediate fusion of the viral envelope with the endosomal membrane. Membrane fusion results in the release of the helical nucleocapsid into the cytoplasm.

In contrast to picornaviruses, the genomic RNA of rhabdoviruses cannot serve as a template for protein synthesis. Consequently, the first biosynthetic process initiated following release of the nucleocapsid is transcription of the genomic RNA into translatable mRNAs. Positive strand RNA viruses such as the picornaviruses do not package their RdRp enzyme as a structural component of the virus particle as their genome can be readily translated to produce the enzyme components soon after entry into the cytoplasm. In contrast, negative strand RNA viruses
FIGURE 2.9  Single-cell replication cycle of a representative rhabdovirus (vesicular stomatitis virus, VSV). The virion binds to a cellular receptor and enters the cell via receptor-mediated endocytosis (1). The acidic environment of the endosome lumen induces conformational changes in the spike glycoproteins which in turn mediate fusion between the viral envelope and the endosome membrane. Membrane fusion releases the alpha helical viral nucleocapsid into the cytoplasm of the host cell (2). The nucleocapsid consists of the (−) strand RNA coated throughout its length with nucleocapsid proteins and a small number of L and P proteins, which catalyze viral RNA synthesis. The (−) strand RNA serves as the template for transcription of five subgenomic mRNAs by the L and P proteins (3). The mRNAs encoding the N, P, M, and L proteins are translated by free cytoplasmic ribosomes (4), while the mRNA encoding the G protein is translated by ribosomes bound to the endoplasmic reticulum (5). Newly synthesized N, P, and L proteins participate in viral RNA replication. This process begins with synthesis of a complementary full-length (+) strand, which is also in the form of a ribonucleoprotein containing the N, L, and P proteins (6). This RNA in turn serves as a template for the synthesis of progeny (−) strand RNAs in the form of nucleocapsids (7). Some of these newly synthesized (−) strand RNAs are used as templates for additional transcription of mRNAs (8). Newly synthesized G proteins enter the secretory pathway (9), where they are glycosylated, oligomerized, and transported to the plasma membrane (10). Progeny nucleocapsids and M proteins are transported to the plasma membrane (11 and 12), where association with regions containing the G proteins initiates assembly and budding of progeny virions (13). From Flint, S.J., Enquist, L.W., Racaniello, V.R., Skalka, A.M., 2008. Principles of Virology, third ed., vol. 1, p. 534. Copyright © Wiley (2008), with permission.
such as the rhabdoviruses do package their RdRp enzyme within the virus particle because the synthesis of viral proteins cannot proceed until the viral genome has been transcribed into mRNAs, and no host cell enzyme capable of performing this function is available in the cytoplasm. The rhabdovirus RdRp enzyme is a multisubunit complex consisting of the large (L) protein, which possesses the catalytic activity of the complex, and the phosphoprotein (P) which functions as an essential, but noncatalytic cofactor. The RdRp complex enters the cytoplasmas a component of the nucleocapsid. The genetic organization of the genomic RNA is highly conserved among the different rhabdoviruses. The 3'-terminal sequences encode for a short nontranslated RNA (“leader”), followed by the coding sequences for 5 genes in the order of N, P, matrix (M), G, and L, and concludes with the 5'-terminal sequences that encode a short, nontranslated “trailer” RNA. Each of these sequences is separated from adjacent sequences by a short, highly conserved intergenic region that plays an important role in transcription as explained below. The ability of the RdRp enzyme to utilize the genomic RNA as a template for transcription or replication is dependent upon two critical parameters. First, the RdRp complex can only access the genomic RNA via the highly conserved 3'-terminal sequences, thus the transcription and replication processes only initiate at this site. Second, the RdRp can only access and utilize viral RNA that is complexed with N protein; naked RNA cannot serve as a functional template for any viral process mediated by the RdRp. Transcription of the viral nucleocapsid results in the synthesis of a series of capped, polya- denylated monocistronic mRNAs, which is achieved as follows. Transcription is initiated at the 3' end and continues until the RdRp enzyme enters the first intergenic region. Each intergenic region contains a sequence that signals the end of transcription of the upstream gene and a sequence that signals the start of transcription of the downstream gene. After the RdRp encounters the first intergenic region it stops transcribing and releases the short leader RNA. The RdRp then scans to the next transcription start signal and begins transcription of the first gene (N gene). In addition to functioning as an RdRp, the L protein has capping activity and will synthesize a methyalted 5' cap structure on the nacent mRNA. When the RdRp encounters the next intergenic region it will pause over a short poly-uridine tract and through a process involving iterative slippage, will use these residues repetitively to synthesize a long poly-adenosine sequence before releasing the mRNA (now containing both a methyalted 5' cap and a poly A tail). This process will be repeated until individual mRNAs representing each viral gene have been transcribed. Reinitiation of transcription following release of an mRNA is an error prone process and some RdRp complexes detach from the template before successfully reinitiating transcription of the downstream gene. RdRp complexes that do detach from the template are unable to re-access the template at an internal site and must reinitiate transcription at the 3' end of negative strand. This situation results in transcriptional attenuation, in which the gene nearest the 3' end of the genome (N gene) is transcribed at the highest level, and transcription of downstream genes decreases progressively with transcription of the L gene (5' terminal gene) occurring at the lowest level.

Replication of the viral genome requires a full-length positive sense RNA that can serve as template for synthesis of the negative sense genomic RNA. Each of the viral mRNAs is of subgenomic length, thus none of these RNAs can serve this purpose. As protein synthesis progresses, a full-length plus strand (antigenome) of viral RNA is produced and this RNA is used in the process of genome replication. The switch from transcription of monocistronic mRNAs to synthesis of genome-length positive strands appears to occur once the cytoplasmic concentration of N protein reaches a critical threshold level. Viral mRNAs are devoid of protein, but the full-length plus and minus strand RNAs are bound throughout their length by repeating copies of N protein. The N protein is an RNA binding protein and is maintained in a soluble, RNA-free form through an association with dimers of the P protein. Once a sufficient level of N protein is achieved, N protein is transferred from the soluble N/P2 complexes onto the nascent leader RNAs as soon as they are synthesized by the RdRp. Additional N proteins will continue to bind to the RNA as it is synthesized. The presence of N protein on the nascent RNA has a profound effect on RdRp function which under these conditions is unaffected by the regulatory signals of the intergenic regions and continues to synthesize a full-length positive strand. The full-length positive strand RNA (in complex with N protein) in turn serves as the template for synthesis of full-length negative strand RNAs. The newly synthesized negative strand RNAs can serve as templates for more mRNA (secondary transcription), as templates for replication, or as genomes for incorporation into progeny virions.

Maturation of rhabdoviruses occurs by budding of newly forming virions through the plasma membrane of the host cell. This process requires specific interactions between components of the three major structural elements of the virus particle; specifically, the G protein-containing envelope, the matrix, and the nucleocapsid. Budding of virus particles occurs through regions of the plasma membrane that contain a high concentration of G protein. The G proteins are synthesized in association with the rough endoplasmic reticulum and are transported to the plasma membrane through the exocytotic pathway.
where they become concentrated in so-called membrane microdomains. The M protein is initially synthesized as a soluble monomer but as the infection proceeds many copies of the M protein localize to the cytoplasmic side of the plasma membrane where they too assemble into M-rich membrane microdomains. Nucleocapsids interact with the M proteins in the microdomains through noncovalent interactions between N and M proteins. In the case of vesicular stomatitis virus, the M protein appears to be primarily responsible for driving the actual budding process, but this is thought to be enhanced by interactions between M proteins and the cytoplasmic portion of the G proteins. Though not detailed here, the budding process also requires functions provided by cellular proteins. In simple terms, virion budding involves the association of the internal components of the virus (nucleocapsid and matrix) with the G-rich membrane microdomains, evagination of the plasma membrane at these sites, and eventual membrane scission.

Rhabdoviruses produce RNA molecules that are functional ligands for several different cellular pattern recognition receptors [PRRs, eg. retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation-associated protein 5 (MDA5), and toll-like receptor 7 (TLR7)], and their recognition can stimulate a type I interferon response by the host cell (see Chapter 4: Antiviral Immunity and Virus Vaccines). For example, the leader RNA that is produced during the transcription process and the full-length genome and antigenome RNAs possess a 5′ triphosphate, and these uncapped RNAs serve as ligands for RIG-I. In addition, viral RNA of positive or negative sense can be bound by TLR7 following delivery of viral products to the endosome as occurs during autophagy. Rhabdoviruses are sensitive to the antiviral effects of type I interferons; however, like the picornaviruses, rhabdoviruses have evolved strategies for inhibiting this innate antiviral defense system of the host cell. Inhibition of the interferon system by vesicular stomatitis virus is mediated by the M protein which limits the synthesis of type I interferon and interferes with the products of interferon stimulated genes (ISGs) by globally suppressing the transcription of host cell genes and by inhibiting the export of cellular mRNAs out of the nucleus. Rabies virus has evolved an alternative strategy for interfering with the interferon response that is mediated by the P protein. The P protein interferes with the RIG-I signaling pathway which prevents activation of the type I interferon genes. In addition, rabies virus P protein inhibits the nuclear localization of phosphorylated STAT 1 and STAT 2 proteins, which limits activation of ISGs and subsequent establishment of the antiviral state [by proteins such as protein kinase R (PKR) and 2′–5′ oligoadenylate synthetase (OAS) as described in detail in Chapter 4, Antiviral Immunity and Virus Vaccines].

**Retroviruses**

The **Retroviridae** family includes pathogens of both humans and animals (see Chapter 14: **Retroviridae**). The retrovirus particle is enveloped and contains envelope-associated glycoprotein spikes. The spike is a multiprotein structure that consists of transmembrane (TM) subunits and surface (SU) subunits. The TM and SU subunits associate with one another to form heterodimers. Three identical TM/SU heterodimers then assemble to form the functional trimetric spike. The interior structures of the virion include a matrix that underlies the envelope and is constructed from repeating copies of the matrix protein (MA), a capsid that is constructed from repeating copies of the capsid protein (CA), and two RNA-based nucleocapsids. The RNA components of the nucleocapsids are identical and consist of single stranded, positive sense RNA (retroviruses are diploid for every virus gene). Each RNA is capped at its 5′ end, contains a poly A tail and is complexed throughout its length by multiple copies of the nucleocapsid protein (NC). Although retroviruses are technically positive strand RNA viruses, their replication cycle is markedly different from that of other positive strand RNA viruses such as the picornaviruses that were discussed earlier. The following description of the retrovirus replication cycle, and depicted in Fig. 2.10, is based on that of a simple retrovirus, and some details will not apply to all members of the **Retroviridae** family.

Infection of a cell by a retrovirus begins with virus binding to receptors (and to coreceptors in some instances) on the host cell. In general, receptor binding is mediated by the SU component of the spike. Most retroviruses appear to enter the host cell at the plasma membrane and no change in pH is required to initiate or complete this process. However, some retroviruses appear to enter host cells via receptor-mediated endocytosis in a manner similar to that described for the rhabdoviruses. For retroviruses that enter the cell at the plasma membrane, receptor binding stimulates conformational changes in the SU subunit, which in turn induce conformational changes in the TM subunit that then mediates fusion between the virus envelope and the plasma membrane. Membrane fusion causes the loss of the envelope, disassembly of the matrix, and release of the virus core. The core consists of the capsid, the nucleocapsids and two viral enzymes [reverse transcriptase (RT) and integrase (IN)] that are required early in the infection process.

Although the details of the next step in the infection process are not entirely understood, evidence suggests that the core undergoes structural changes, probably mediated by cellular proteins, and these structural changes are required to initiate the process of reverse transcription by the core-associated RT enzyme. RT is a multifunctional enzyme that possesses RNA-dependent DNA
FIGURE 2.10 Single-cell replication cycle of a simple retrovirus. The virus attaches by binding of the viral envelope protein to specific receptors on the surface of the cell (1). The viral core is deposited into the cytoplasm following fusion of the viral envelope with the plasma membrane (2). Entry of some beta- and gammaretroviruses may involve endocytic pathways. The viral RNA genome is reverse transcribed by the virion reverse transcriptase (RT) within a subviral particle (3). The product of reverse transcription is a linear, double stranded, complementary DNA (cDNA) with ends (long terminal repeats, LTRs) that are shown juxtaposed in preparation for integration. Viral DNA and the integrase (IN) enzyme gain access to the nucleus with the help of intracellular trafficking machinery or, in some cases, by exploiting nuclear disassembly during mitosis (4). Integrative recombination catalyzed by IN results in insertion of the viral cDNA into a host cell chromosome, which establishes the provirus (5). Transcription of the proviral DNA by RNA polymerase II produces full-length RNA transcripts (6). Some full-length transcripts are exported from the nucleus and serve as mRNAs (7), which are translated by cytoplasmic ribosomes to form the viral Gag and Gag—Pol polyprotein precursors (8). Some full-length transcripts which are destined to become encapsidated as progeny viral genomes associate into dimers (9). Other full-length transcripts are spliced within the nucleus before being exported to the cytoplasm (10). These spliced mRNAs encode the Env polyprotein precursor and are translated by ribosomes bound to the endoplasmic reticulum (11). The Env glycoproteins are transported through the Golgi apparatus where they are processed and eventually cleaved by a cellular enzyme to form the mature SU—TM spike complex (12). Mature envelope proteins are delivered to the surface of the infected cell (13). Internal virion components (viral RNA, Gag and Gag—Pol precursors) assemble at budding sites containing the viral spikes (14). Type C retroviruses (eg, alpharetroviruses and lentiviruses) assemble at the inner face of the plasma membrane, as illustrated. Other types (A, B, and D) assemble on internal cellular membranes. The nascent virions bud from the surface of the cell (15). Maturation (and infectivity) requires the action of the virus-encoded protease (PR), which is itself a component of a core precursor polyprotein (Gag—Pol in the model represented here). During or shortly after budding, PR cleaves the Gag and Gag—Pol precursors at specific sites to yield the individual viral proteins (16). This process yields functional forms of RT and IN, and frees the NC, CA, and MA proteins to assemble into the internal structures of the virion (eg, nucleocapsids, capsid, and matrix). From Flint, S.J., Enquist, L.W., Racaniello, V.R., Skalka, A.M., 2008. Principles of Virology, third ed., vol. 1, p. 531. Copyright © Wiley (2008), with permission.
polymerase (RdDp) activity. RT is a primer-dependent polymerase and uses the viral single stranded RNA as a template to synthesize a linear, complementary double stranded DNA (cDNA) product. The primer used to initiate the synthesis of DNA is a cell-derived tRNA that is base paired to a primer binding site on the RNA. This tRNA was acquired from the cell that generated the virus particle and it enters the newly infected cell already bound to the viral RNA. The molecular details of the reverse transcription process are not presented here, but three important outcomes of the process should be noted. First, the viral RNA is degraded in the process, thus the viral gene segments, which are fully capable of functioning as mRNA, are never translated into proteins. Second, the process causes the duplication and transposition of specific viral sequences which together result in the formation of repeated sequences at the termini of the cDNA. These direct repeats are referred to as the long terminal repeats (LTRs) and they perform important replication-related functions as described below. Third, upon completion of the reverse transcription process the cDNA product exists as a component of a nucleoprotein complex called the preintegration complex (PIC). The complex also contains the virus protein (eg, the IN enzyme) and cellular proteins that are now poised to mediate the integration of the cDNA into a chromosome of the host cell if access to cellular DNA can be achieved.

Most retroviruses are not able to transport the PIC into the nucleus, and therefore, these viruses can only integrate their cDNA into a chromosome of an actively dividing cell as cell division involves the temporary dissolution of the nuclear membrane. Retroviruses belonging to the genera Lentivirus and Spumavirus have evolved mechanisms for transporting their PIC into the nucleus that make it possible for these viruses to integrate their cDNA into chromosomes of nondividing cells. The integration reaction is initiated by IN which cleaves host cell DNA at the site selected for integration, and the process is dependent on interactions between IN and the LTR sequences of the cDNA. The final steps of the integration process are performed by cell-derived DNA repair enzymes. Integration of the cDNA into a host cell chromosome is essentially random and does not require specific host DNA sequences, but integration generally occurs within regions of a chromosome that are transcriptionally active, and consequently, more readily accessible. The integrated viral cDNA is referred to as the provirus, and establishment of the provirus must be achieved before the expression of viral genes can occur.

The DNA sequences that control transcription of the viral genes are located within the LTRs. These sequences are similar to those that regulate the expression of cellular genes (eg, TATA box, binding sites for cellular transcription factors, etc.). Therefore, the LTR sequences are accessible to the transcription machinery of the host cell and viral transcripts are synthesized by cellular RNA pol II, capped by cellular capping enzymes, and polyadenylated by the cellular poly A polymerase enzyme. Transcription is initiated within the LTR that is positioned upstream of the viral genes and continues through the entire provirus sequence. A polyadenylation signal encoded by the downstream LTR is utilized for the addition of a poly A tail. Due to their sequence similarity, both LTRs are capable of initiating transcription; however, transcription activity initiated by the upstream LTR typically interferes with the initiation of transcription from the downstream LTR. This phenomenon, which is referred to as promoter occlusion, normally prevents the downstream LTR from initiating transcription of downstream cellular sequences.

The single viral mRNA contains the sequence of all viral genes in the order of gag (encoding MA, CA, NC proteins, and the protease (PR) enzyme), pol (encoding the RT and IN enzymes), and env (encoding the glycoprotein precursor of SU and TM). In some retroviruses, the PR enzyme in encoded in the pol region. Depending on the particular retrovirus, the open reading frames that encode Gag, Pol and Env can be in frame with one another or out of frame, and the open reading frames that encode Gag and Pol can be continuous or overlapping. The capped and polyadenylated transcripts produced by the simple retroviruses experience one of two alternative fates. If the transcript is exported from the nucleus without being spliced it will serve as a transcript for the synthesis of the Gag polypeptide (encoding only MA—NC—CA—PR) and/or a larger Gag—Pol polyprotein (encoding MA—NC—CA—PR—RT—IN). The Gag open reading frame terminates with a stop codon; and therefore, the majority of ribosomes that translate the unspliced transcript will only synthesize the Gag polypeptide. However, a small percentage of translating ribosomes will synthesize the larger Gag—Pol polyprotein using one of two mechanisms. If the Gag and Pol open reading frames are continuous and in frame then the Gag—Pol polyprotein can be produced if the translating ribosome reads through the Gag stop codon. This process, in which the ribosome treats the stop codon as a sense codon, is referred to as stop codon suppression. If the Gag and Pol open reading frames are overlapping and out of frame, then the Gag—Pol polyprotein can be produced if the translating ribosome shifts from its original reading frame (the Gag reading frame) into the Pol reading frame. The ribosomal frame shift is facilitated by sequences within gag and occurs just upstream of the Pol open reading frame. Typically, the Gag and Gag—Pol polyproteins are not resolved into their individual protein components within the cell, but instead only undergo proteolytic processing after they have been incorporated into progeny virions during the virus assembly process. This process will be described in more detail below.
Alternatively, the transcript can be spliced prior to being exported to the cytoplasm. Splicing removes an intron that includes the sequences encoding Gag and Pol; therefore, spliced transcripts only retain the Env open reading frame and can only be translated into the Env glycoprotein that serves as the precursor to SU and TM. Translation of this transcript occurs in association with the endoplasmic reticulum and the Env glycoproteins are processed and routed to the cell surface using the endoplasmic reticulum/Golgi apparatus protein export system. The Env glycoprotein is cleaved into its SU and TM components by the host cell enzymes called furin (or by a furin-like enzyme) during its transits through the trans-Golgi or after its arrival at the cell surface. Splicing of viral transcripts is not unique to the retroviruses. For example, splicing of viral transcripts occurs during the replication of influenza A virus (Family Orthomyxoviridae), Borna disease virus (Family Bornaviridae), and of most DNA viruses.

In addition to functioning as mRNA for the production of the Gag and Gag–Pol polyproteins, the unspliced transcript can also be incorporated into progeny virions as genomic RNA. These full-length RNAs possess a packaging signal located within the Gag sequence. Packaging signals of two RNAs interact with one another, facilitating the formation of RNA:RNA dimers. The spliced viral mRNAs lack this sequence and are unable to participate in dimer formation. Similar to the rhabdoviruses, formation of the retrovirus particle generally requires specific and coordinated interactions between components of the three major structural elements of the virus particle. With respect to retroviruses these structural elements include the spike-modified membrane microdomains, the Gag and Gag–Pol polyproteins, and the dimeric RNAs. Key interactions responsible for virion assembly and budding include those that take place between the MA-component of the polyproteins and the cytoplasmic tails of TM (and with the membrane), and those that take place between the NC-component of the polyproteins and the dimeric RNA. These interactions help to drive the budding process by which immature virions are formed. The newly budded immature virions contain the Gag and Gag–Pol polyproteins and lack defined internal structures such as a matrix, capsid, or nucleocapsid. To this point in the process, the PR enzyme has been inactive; however, soon after formation of the immature virion, the PR enzyme is activated and proceeds to process the Gag and Gag–Pol polyproteins into their individual constituent proteins. Once released, these proteins then assemble into the matrix, capsid, and nucleocapsid structures that are characteristic of the mature, infectious virus particle. Proteolytic processing of the Gag–Pol polyproteins also releases RT and IN which are now available to perform the early replication events required to infect the next cell.

**Adenoviruses**

Adenoviruses belong to the family Adenoviridae (see Chapter 10: Adenoviridae). Unlike picornaviruses, rhabdoviruses and retroviruses, the adenovirus genome consists of DNA. The adenovirus particle is nonenveloped and consists of an icosahedral capsid that is constructed from hexon (trimers of protein II) and penton (pentamers of protein III) subunits. Prominent structures called fibers (trimers of protein IV) are associated with the penton subunits and project outward from each of the icosahedron’s 12 vertices. The adenovirus particle also has numerous proteins located internally; some of which are in contact with the penton and hexon subunits, and others that are associated with the DNA genome. The genomic DNA consists of 30–36 kbp of linear dsDNA, contains terminal repeat sequences that play an important role in the DNA replication process, and is covalently bound to a virus-encoded protein (terminal protein) at each 5’ end.

The following description of the adenovirus replication cycle, and the representation of the process that is depicted in Fig. 2.11, is based on that of human adenovirus 2. The initial interaction of adenovirus with a host cell is mediated by the fibers that bind to a host cell protein called the coxsackievirus and adenovirus receptor (CAR). High-affinity binding between the fiber and this receptor allows the penton base proteins to make contact with cellular integrins, whose normal function is to bind the host cell to components of the extracellular matrix. This binding initiates the process of clathrin-mediated entry with subsequent internalization of the virion into clathrin-coated pits, and initiates the first steps of virion uncoating. The interactions that occur between the fibers and CARs and between pentons and integrins, and perhaps other factors that are not yet adequately characterized, induce substantial changes in the capsid structure. These changes include the shedding of the fibers and externalization of a lytic factor (protein VI) from the virion interior into the endosome lumen. Protein VI mediates disruption/fragmentation of the endosomal membrane which allows the modified capsid to enter the cytoplasm. After release from the endosome the virions associate with the molecular motor dynein which then transports them along microtubules to a nuclear pore. At the nuclear pore the capsid establishes interactions with a number of host cell proteins, including the nuclear pore filament protein Nup214, kinesin-1, and histones, which further destabilize the virion structure and result in the release of the viral DNA into the nucleus.

Gene expression programs of most DNA viruses are temporally regulated with specific genes being expressed at different times. The expression of adenovirus genes occurs in three phases, which are referred to as immediate early, early, and late. The adenovirus genes are arranged.
FIGURE 2.11  Single-cell replication cycle of human adenovirus type 2. The virus attaches to a permissive human cell via interaction between the fiber and (with most serotypes) the Coxsackievirus and adenovirus receptor on the cell surface. The virus enters the cell via endocytosis (1 and 2); a process that depends on the interaction of a second virion protein, penton base, with a cellular integrin protein (red cylinder). Partial disassembly of the virion within the endosome releases a virus protein (protein VI) that disrupts the endosomal membrane and facilitates release of the modified virion into the cytoplasm (3). Following further uncoating, the viral genome associated with core protein VII is imported into the nucleus (4). The host cell RNA polymerase II system transcribes the immediate-early E1A gene (5). The mRNAs are alternatively spliced and then exported to the cytoplasm (6), where they are translated into multiple, related E1A proteins (7). The E1A proteins are imported into the nucleus where they regulate transcription of both cellular and viral genes (8). The larger E1A protein stimulates transcription of the viral early genes by cellular RNA polymerase II (9a). Transcription of the VA genes by host cell RNA polymerase III also begins during the early phase of infection (9b). The early pre-mRNA species are processed, exported to the cytoplasm (10), and translated (11). These early proteins include the viral replication proteins, which are imported into the nucleus (12) and cooperate with a limited number of cellular proteins in viral DNA synthesis (13). Replicated viral DNA molecules can serve as templates for further rounds of replication (14) or for transcription of late genes (15). The major late promoter is activated by viral DNA replication, but maximal efficient transcription requires the late IVa2 and L4 proteins. Processed late mRNAs are selectively exported from the nucleus as a result of the action of the E1B 55-kDa and E4 Orf6 proteins (16). Efficient translation of the late transcripts requires the virus-expressed VA RNA-1 and the late L4 100-kDa protein (17). The latter protein also serves as a chaperone for assembly of trimeric hexons as they and the other structural proteins are imported into the nucleus (18). Within the nucleus, capsids are assembled from these proteins and the progeny viral genomes to form noninfectious immature virions (19). Assembly requires a packaging signal located near the end of the genome, as well as the IVa2 and L4 22/33-kDa proteins. Immature virions contain the precursors of the mature forms of several proteins. Mature infectious virions are formed (20) when these precursor proteins are cleaved by the viral L3 protease, which enters the virion core. Progeny virions are released (21), usually upon destruction of the host cell via mechanisms that are not well understood. From Flint, S.J., Enquist, L.W., Racaniello, V.R., Skalka, A.M., 2008. Principles of Virology, third ed., vol. 1, p. 504. Copyright © Wiley (2008), with permission.
in sets called transcription units. Each transcription unit is controlled by a single promoter that is used by the transcriptional machinery of the host cell, and polyadenylation signals define the 3’ ends of the viral transcripts. Each transcription unit directs the synthesis of a single primary RNA; however, alternative splicing yields a population of mRNAs that encode multiple different proteins. The first transcription unit to become transcriptionally active is E1A which encodes the immediate early proteins. The E1A primary transcripts are alternatively spliced to form transcripts that encode a family of E1A proteins. The major E1A proteins (289R and 243R) perform several critical functions that are required during the initial stages of the infection. First, the E1A proteins interfere with the type I interferon response as will be discussed below. Second, they induce the host cell to enter the S phase of the cell cycle by directly interacting with the retinoblastoma (Rb) tumor suppressor protein. Adenoviruses typically infect terminally differentiated cells that are not actively dividing. By inducing the host cell to enter the S phase the virus creates a cellular environment that is more conducive to replication of the viral DNA. The major E1A proteins also activate the transcription units for the early genes, as well as activate some cellular promoters. Collectively, the proteins expressed from the early genes perform three major functions; including the inhibition of apoptosis, replication of viral DNA, and inhibition of host immune defenses.

Two proteins expressed from the E1B transcription unit (E1B-19K and E1B-55K) inhibit apoptosis, which is a normal cellular response to unscheduled entry into the S phase (as induced by E1A) and to cellular stress induced by virus infection. The E1B-19K protein is a homolog of the antiapoptotic cellular protein Bcl-2. Like Bcl-2, E1B-19K binds to the pro-apoptotic protein Bax and inhibits its ability to mediate mitochondrial release of cytochrome C, which is a potent inducer of the intrinsic apoptosis pathway (see Chapter 3: Pathogenesis of Viral Infections and Diseases). The E1B-55K protein induces the rapid turnover of the tumor suppressor protein called p53 which becomes stabilized in the infected cell as a consequence of E1A-mediated inactivation of Rb. Under normal conditions, stabilized p53 activates transcription of cellular genes that cause cell cycle arrest (eg, p21) and of genes such as Bax, which promote apoptosis.

Three proteins expressed from the E2 transcription unit cooperate to replicate the viral DNA. The precise details of the genome replication process will not be addressed here, but the general functions of these three proteins will be described. One of these proteins is the DNA polymerase that catalyzes the replication of the DNA. The second protein is the preterminal protein (Pre-TP) which serves as a primer for DNA replication in much the same way as VPg served as a protein primer for replication of the picornavirus RNA genome, except that no cis-acting replication-like element is required. At the conclusion of the DNA replication process a Pre-TP remains covalently attached to each 5’ end of the DNA. Later in the replication process as DNA genomes are incorporated into newly assembling virions, Pre-TP is cleaved by a virus-encoded protease into a smaller form called the terminal protein (TP). The third E2 protein is the DNA binding protein (DBP) which binds to the single stranded DNA that is displaced from the dsDNA template during the replication process. After being displaced and bound throughout its length by DBP, the ssDNA serves as a template for the synthesis of a genome-length dsDNA.

The final transcription unit to become active is that which controls expression of the late genes. The late genes encode the major structural proteins of the virus and nonstructural proteins that function in the virus assembly process. Late gene expression does not begin until after the onset of DNA replication and it is enhanced by a virus-encoded protein called IVa2. Transcription of the late genes is controlled by the major late promoter which defines the 5′ end of all late mRNAs. The 3′ end of late transcripts is determined by any one of 5 different polyadenylation signals that are present within the primary transcript. The use of alternative polyadenylation signals leads to the production of a nested set of five different transcripts, some of which retain one or more internal polyadenylation sites. The polyadenylated transcripts are then alternatively spliced into multiple unique transcripts, each of which is then translated into a different late protein. The late proteins are then transported to the nucleus where they participate in the virion assembly process. Unlike the picornviruses which possess a simple icosahedral structure, the adenovirus virion is a large and complex structure. Simple self-assembly models cannot account for this degree of complexity. Accordingly, viral proteins have been identified that act as chaperones for moving structural proteins to maturation sites and others that act as scaffolds for assembling the virion subunits. A virus-encoded protease that requires DNA as a cofactor to prevent premature proteolysis participates in the maturation process by degrading scaffold proteins and cleaving precursor proteins. Late in infection, inclusion bodies composed of large crystalline arrays of newly assembled virions appear in the nucleus of the host cell. Release of progeny virions occurs following lysis of the host cell.

As with other viruses, adenovirus infections are detected by microbial pattern recognition receptors (PRRs) of the host cell and infection initiates a type I interferon response (see Chapter 4: Antiviral Immunity and Virus Vaccines). However, adenoviruses actively limit the effectiveness of the response in several ways. First, the E1A proteins inhibit the activity of a cellular
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The external capsid structure of virtually all nonenveloped animal viruses consists of an icosahedron, and these exist in variable levels of complexity. For the structurally simple icosahedral viruses such as those belonging to the Parvoviridae, Polyomaviridae, Papillomaviridae, and Picornaviridae families, the structural proteins spontaneously associate into the repeating structural subunit of the capsid called the protomer. A defined number of protomers are then used to assemble the mature icosahedral capsid. The protomers of some nonenveloped viruses are able to assemble into capsids in the absence of the genomic molecule (eg, canine parvovirus and human papilloma virus); for other viruses the genome appears to serve as the nucleation site for protomer assembly and capsids only form in the presence of a genomic molecule (eg, SV40, Polyomaviridae). The structure of some icosahedral viruses is more complex and does not consist of just a single type of repeating subunit. For example, the external structure of the adenovirus particle is assembled from individual hexamer subunits, pentamer subunits and trimeric fibers. Assembly of the adenovirus particle requires chaperone proteins that facilitate the proper assembly and folding of other structural components of the virus particle, and scaffold proteins that serve as temporary components of intermediate structures that are generated during the assembly process. The proteins that serve as chaperones and/or scaffolds can be displaced during the assembly process and may not remain as a structural component of the mature virion. In addition, cleavage of some virion-associated proteins is required to convert the intermediate structures into the mature, infectious form of the virus particle. Nonenveloped viruses that contain an RNA genome replicate and complete their assembly process in the cytoplasm of the host cell. Nonenveloped viruses that contain a DNA genome typically replicate and complete their assembly process in the nucleus. Most nonenveloped viruses are released only when the cell lyses, thus for these viruses the processes of virus assembly and virus release are separate and sequential events.

Enveloped viruses acquire their envelope as the internal structures of the virus (eg, nucleocapsid(s) and matrix components) bud through a cellular membrane. Depending on the virus, budding can occur at the plasma membrane, through the membranes of the endoplasmic reticulum or Golgi apparatus, or through the inner membrane of the nucleus. For most enveloped viruses budding occurs at regions of the membrane in which cellular glycoproteins have for the most part been displaced by the glycoproteins of the virus (Fig. 2.12). This ensures that the viral glycoproteins are incorporated into the virion during the budding process. However, displacement of host cell glycoproteins is not an absolute requirement and some viruses (eg, human immunodeficiency and vesicular stomatitis viruses) readily incorporate host cell glycoproteins into virus particles during budding. The viral glycoproteins typically associate into oligomers (usually homotrimers or homotetramers) to form the spike (peplomer) structures. Viral glycoproteins typically consist of a hydrophilic domain projecting outward from the membrane, a hydrophobic transmembrane domain, and a short hydrophilic domain projecting into the cytoplasm (or virion interior).

In general, the icosahedral nucleocapsids (eg, togviruses and flaviviruses) and helical nucleocapsids (eg, orthomyxoviruses and rhabdoviruses) of an enveloped virus assemble prior to budding. These preformed nucleocapsids then localize to the appropriate cellular membrane
and participate in the budding process. In the relatively rare case of an enveloped virus with icosahedral symmetry (e.g., togaviruses), each nucleocapsid protein (C protein) interacts directly with the cytoplasmic domain of a single membrane glycoprotein (E2), and these interactions help drive the budding process. For viruses that possess helical nucleocapsids, the budding process tends to be driven by interactions between the matrix proteins and the cell membrane and/or the surface glycoproteins, and between matrix proteins and the proteins of the nucleocapsid. For some viruses, the energy and forces that are required to induce curvature of the membrane and eventual membrane scission is provided by protein:protein and protein:lipid interactions mediated by virus constituents alone. However, many enveloped viruses utilize proteins of the cellular endosomal sorting complexes required for transport (ESCRT) system to assist in the budding process. One of the normal functions of the ESCRT proteins is to catalyze the budding of membrane-bound vesicles into the endosome to form multivesicular bodies. This process is physiologically similar to the budding of a virus with respect to the process being initiated on the cytoplasmic side of a membrane and the product of membrane scission (a vesicle or a virion) being formed on the extracytoplasmic side of the membrane. Depending on which proteins of the ESCRT system are involved, these proteins assist in the virus budding process itself, and/or in the final step of membrane scission which is required to release and fully envelop the virus. Viruses that acquire their envelope from an internal membrane enter the secretory pathway of the cell and are transported in exocytotic vesicles to the cell surface where they are released upon fusion of the vesicle with the plasma membrane (exocytosis) (Fig. 2.12). For these viruses the processes of virus assembly and virus release are separable and occur in sequence. Viruses that bud through the plasma membrane are released directly into the extracellular environment, thus, for these viruses the processes of virus assembly and release occur simultaneously and are essentially inseparable.

Many glycoproteins encoded by enveloped viruses are synthesized as precursor proteins than are subsequently processed by site-specific proteolysis before or after being incorporated into the mature virion. This is particularly common for glycoproteins that mediate the process of membrane fusion during cell entry. Cleavage of the precursor is most commonly performed by the cellular enzyme called furin (or a furin-like protease), which is an ubiquitously expressed endoprotease that resides in the trans-Golgi compartment and at the cell surface. Furin cleaves its substrates on the carboxyl side of a B–X–B–B sequence motif (B represents Arg or Lys and X represents a nonspecified residue). The precursor of the hemagglutinin glycoprotein of influenza A virus (HA0) is not cleaved by furin, but instead is cleaved into the functional subunits of the HA spike (HA1 and HA2) after the newly budded virion has been released from the host cell. HA0 is cleaved by trypsin-like enzymes present in secretions of the respiratory tract of humans and the gastrointestinal tract of the avian host. This finding was a major discovery in the early 1970s that allowed the routine propagation of influenza virus viruses in cell culture (in

FIGURE 2.12 Maturation of enveloped viruses. (A) Viruses that possess a matrix (and some viruses that lack a matrix) bud through a patch of the plasma membrane in which glycoprotein spikes (peplomers) have accumulated over matrix proteins. (B) Most enveloped viruses that lack a matrix bud into cytoplasmic vesicles (rough endoplasmic reticulum or Golgi), pass through the cytoplasm in smooth vesicles, and are released from the cell by exocytosis.
which trypsin is added to the growth medium). In general, cleavage of the precursor converts a protein that is not functional for membrane fusion, into its fusion-competent form. Cleavage of the precursor primes the virus to perform the entry and uncoating processes in response to proper stimuli (as discussed earlier), and is generally required to produce an infectious virus particle.

Influenza virus also depends on a second enzymatic activity in order to be efficiently released from the host cell and to prevent aggregation of virus particles. The HA spike of influenza A virus binds sialic acid and uses this carbohydrate moiety as a receptor for attaching to host cells. However, proteins that comprise the HA and neuraminidase (NA) spikes also possess sialic acid, thus newly released virions are inclined to bind to the host cell from which they budded, and to neighboring virions. The enzymatic activity of the NA spike inhibits these nonproductive binding events by cleaving sialic acid from the cell surface and from the virion itself. The drug called oseltamivir (Tamiflu) inhibits the enzymatic activity of NA, which causes virion aggregation and restricts cell to cell spread.

It should be noted that for most viruses (eg, togaviruses, herpesviruses, and retroviruses) incorporation of genomic molecules into the capsid or nucleocapsid structure is highly selective and is dependent of the presence of a highly conserved sequence called a packaging signal that is only present in the appropriate genomic RNA or DNA molecules. In contrast, other viruses (eg, rhabdoviruses and parvoviruses) are not highly selective with respect to the nucleic acid that is packaged and viral nucleic acids of both polarity (genomic and antigenomic) are packaged into virions.

Unlike most other cell types in the body, epithelial cells display polarity, which means that they possess an apical surface that interfaces with the external environment (eg, lumen of respiratory tract or gastrointestinal tract) and a basolateral surface that interfaces with underlying cells. These surfaces are chemically and physiologically distinct. Viruses that are shed to the exterior (eg, influenza A virus) tend to bud from the apical plasma membrane, whereas other viruses (eg, C-type retroviruses) bud through the basolateral membrane, which may enable the virus to enter the bloodstream or lymphatic system as a prelude to establishing systemic infection (Fig. 2.13).

QUANTITATIVE ASSAYS OF VIRUSES

The study of basic virus processes and virus-based diseases often requires the researcher or clinician to know how much virus exists in a given sample. The reproducibility of both in vitro and in vivo experiments depends upon using a consistent amount of virus to initiate an infection. In assessing clinical cases, it may be important to determine the quantity of virus in various tissues or fluids as a part of the determination of pathogenicity and to select the correct specimens for diagnostic testing. A common metric used to assess the effectiveness of antiviral drugs is to compare the viral load (or “burden”) in clinical specimens before and after drug treatment. The answer to the question as to how much virus is present in an individual sample or specimen may not be simple, and is test dependent. There are two general types of viral quantification tests; specifically, biological assays and physical assays. Quantifying virus in a single sample using different assays will often yield different answers, and it is essential to understand the reasons for these differences. Physical assays that do not depend on any biological activity of the virus particle include electron microscopic particle counts, hemagglutination, immunological assays such as antigen-capture enzyme-linked immunosorbent assay (ELISA) tests and, most recently, quantitative PCR assays. Biological assays that depend on a virus particle initiating a successful replication cycle include plaque assays and various endpoint titration methods.
The difference between the amount of virus detected using a physical assay such as particle counting by electron microscopy and a biological assay such as a plaque assay is often referred to as the particle to pfu ratio. In virtually all instances, the number of physical particles exceeds the number determined in a biological assay. For some viruses this ratio may be as high as 10,000:1, with ratios of 100:1 being common (Table 2.2). The reasons for the higher number of physical particles as compared with infectious particles are virus dependent and include: (1) the assembly process is inefficient and error prone, and morphologically complete particles can be formed without the correct nucleic acid component; (2) not all virions that bind a receptor or initiate the entry and uncoating processes are successful in establishing a productive infection; (3) the replication process is highly error prone (RNA viruses), and virus stocks can contain particles with lethal mutations; (4) virus stocks are produced or maintained under suboptimum conditions such that infectious particles are inactivated; (5) tests for infectivity are performed in animals or cells that are not optimum for detecting infectious particles; (6) host cell defenses prevent some infectious particles from successfully completing the replication process. The choice of host or host cell for the biological assays is a critical determinant for defining the amount of infectious virus in a sample. It is not unusual for assays in the natural host animal to provide the highest estimates of infectious units, as available cell cultures may be a poor substitute for the target cells in the animal.

**Physical Assays**

**Direct Particle Counts by Electron Microscopy**

The most direct method to determine the concentration of virus particles in a sample is to visually count the particles using an electron microscope. This process is not performed routinely because it requires expensive equipment and highly trained technicians. In this assay the virus sample is first mixed with a sample of standard particles (eg, latex beads) of known concentration. The virus/standard particle mixture is then observed using an electron microscope, and the numbers of virus particles and standard particles are counted separately. The number of virus particles counted is easily converted into a concentration (eg, virus particles/mL) by multiplying the ratio of the virus particle count/standard particle count by the known concentration of the standard particles. This procedure is most accurate for nonenveloped viruses that produce highly stable virus particles with unique geometric shapes such as picornaviruses, reoviruses, and adenoviruses. This process cannot assess biological activity of the preparation, but it can be used to assess whether the particles contain nucleic acid, as visual observation can be used to differentiate empty capsids from complete particles.

**Hemagglutination**

As mentioned earlier in this chapter, some virus-infected cells acquire the ability to bind red blood cells on their surface (hemadsorption) due to interactions between surface-expressed viral proteins and ligands on the red blood cell. The free virus particles of some viruses are also able to bind to red blood cells, and when mixed together will cause the cells to aggregate into a lattice of cross-linked cells. This property is called hemagglutination, and can be used as the basis for quantifying viruses that possess this activity (eg, influenza A virus). The hemagglutination assay cannot accurately determine the number of virus particles present in a sample (ie, virus particles/mL); but it is useful for comparing the relative concentrations of a virus between samples, such as those obtained from multiple infected hosts, or those collected sequentially from an individual host on different days or times. To perform this assay the virus-containing sample is first processed in a serial twofold dilution series (typically in a 96-well microtiter plate). A solution containing red blood cells is then added to each sample well. After a defined period of time the wells are observed visually for the presence of hemagglutinated red blood cells which appear as a thin continuous layer of cells covering the bottom surface of the well. Nonagglutinated red blood cells settle into a small “button” of cells in the center of the well. The “HA titer” of the stock virus sample is reported as the inverse of the highest dilution that completely agglutinates the red blood cells (Fig. 2.14).

**Quantitative Polymerase Chain Reaction Assays**

With the development of real-time (quantitative) PCR assays (see Chapter 5: Laboratory Diagnosis of Viral Infections), it is now possible to determine the

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**TABLE 2.2 Comparison of Quantitative Assay Efficiency**

| Method                                      | Amount (per mL) |
|---------------------------------------------|-----------------|
| Direct electron microscope (EM) count       | $10^{10}$ EM particles |
| Quantal infectivity assay in eggs           | $10^9$ egg ID<sub>50</sub> |
| Quantal infectivity assay by plaque formation | $10^8$ pfu      |
| Hemagglutination assay                      | $10^3$ HA units |

ID<sub>50</sub>, infectious dose 50; pfu, plaque-forming units; HA, hemagglutination.
concentration of a virus-specific nucleic acid in a test sample. PCR can detect nucleic acid sequences in virtually any context, not just in a virus particle. The increased sensitivity of PCR over virus isolation in many instances is achieved by detecting nonvirion nucleic acid in tissue samples. To use PCR correctly to quantify virus, it is necessary first to treat the suspension with nucleases to degrade all nonvirion nucleic acid. Virion-associated nucleic acid will be protected by the intact virus particle. With copy number controls being included in the assay as a basis for comparison, the concentration of the target nucleic acid in the treated sample can be determined. This type of assay does not detect empty capsids (those that do not contain viral nucleic acid), and it is not influenced by the infectivity of the preparation.

**Biological Assays**

**Plaque Assays**

Perhaps no other procedure in virology has contributed as much to the development of the field as the plaque assay. The plaque assay was originally developed by d’Herelle in 1915–1917, in his initial studies on bacteriophage. The assay is elegantly simple and is the most accurate of the quantitative biological assays. To perform a plaque assay with bacteriophage, the sample is first processed in a serial 10-fold dilution series in a bacterial culture medium. A suspension of host bacteria in a melted culture medium (top agar) is then added to each diluted sample. This mixture is then poured onto a nutrient agar culture plate to distribute the bacteriophage/bacteria suspension evenly across the surface of the plate where it will rapidly cool and solidify. The dishes are placed into an incubator and over time the host bacteria divide and produce a visible “lawn” of bacteria over the surface of the agar plate. Bacteria that are infected with bacteriophage die and release progeny virions which in turn infect and kill neighboring bacteria. Eventually, enough bacterial cells are killed so that a clear area of cell-free agar is observed. These clear areas are referred to as plaques. It is important to note that although an extremely high number of bacteriophage will be present in each plaque, the plaque originated from the infection of one bacterial cell by a single bacteriophage; and therefore, each plaque represents one bacteriophage present in the sample that was plated. No plaques should appear on uninfected control plates. If the original stock sample has a high concentration of bacteria, then the plates containing the low dilution samples will be completely or nearly completely cleared due to most or all of the bacteria being infected and killed. Plates used to assay the very highest dilution samples may have only a few plaques or none at all. Somewhere between these extremes, plates will be identified that contain a number of plaques that can be accurately counted, and these plates will be used to determine the concentration (titer) of the bacteriophage in the original sample. This will be achieved by taking into account the sample dilution, volume tested, and the plaque count. In 1953, the bacteriophage plaque assay was modified for use with the newly developed tissue culture systems and animal viruses. This assay works best with cytopathic viruses that induce the lysis of their host cell. Although variations of the assay exist, in general, it is performed as follows. The stock virus sample is processed in a serial dilution series as described above. Liquid growth medium is removed from plates containing monolayers of cultured cells, and each diluted virus sample is then overlaid onto a monolayer of cells at a standard volume that minimally covers the cells. The plates are then incubated for a short period to allow the virus to bind and enter the cells, and then the cells are overlaid with nutrient agar. The nutrient agar overlay prevents newly produced progeny virions from spreading freely to distal regions of the plate, but it does not restrict the movement of these virions to immediately adjacent cells. Eventually, virus will spread from the original host cell to infect enough neighboring cells to form a focus of infected cells that can be visualized when stained with a vital dye (Fig. 2.15). Both immunofluorescent and immunohistochemical staining procedures have been developed for conducting plaque assays with noncytopathic viruses (Fig. 2.3).
In addition to its use to quantify the amount of virus in a sample, the plaque assay established a fundamental principle applicable to the vast majority of animal viruses; namely that a single virus particle was sufficient to establish a productive infection. This was proven by determining that the number of plaques in an assay increased in a linear fashion when plotted against the dilution factor, that is, the plaque number followed a one-hit kinetic curve. This is not the case for many plant viruses, in which segmented genomes are incorporated into separate virus particles and a productive infection requires coinfection of a cell by multiple viruses. Plaque assays were also instrumental in early studies of viral genetics, as plaque variants either occurring naturally or induced chemically could be selected by isolating virus from individual plaques (biologically cloned) and studied to determine the impact of the mutation on viral growth properties.

**Endpoint Titration Assays**

Before the development of the plaque assay for animal viruses, and for noncytopathic viruses that do not produce plaques, the quantification of virus stocks was achieved by inoculating virus into test animals or embryonated eggs. As with the plaque assay, these assays begin with the serial dilution of the sample or specimen. Each diluted sample is then inoculated into one or more test animals or eggs. A successful infection could be scored directly, being inferred from the death of the animal or egg, or indirectly by confirming an immune response to the virus in the infected host. At low dilutions, all animals would become infected whereas, at high dilutions, none of the animals would become infected. At some intermediate dilution only some of the animals or eggs would show evidence of infection. Two methods were devised (Reed–Muench and Spearman–Karber) to use the results (ie, number of infected vs number of uninfected at each dilution tested) to calculate the dilution of the virus that would infect 50% of the test animals. In this case, the titer of the stock virus would be expressed as an infectious dose 50 (ID$_{50}$) (Table 2.3). If the virus causes the death of the animal or egg, then this assay can be used to determine its lethal dose 50 (LD$_{50}$) or egg infectious dose 50 (EID$_{50}$), respectively. Endpoint titration assays can also be performed in cultured cells and in this version of the assay the titer of the virus is reported as the tissue culture infectious dose 50 (TCID$_{50}$). Although not as accurate as plaque assays and not as amenable to statistical analysis, the TCID$_{50}$ endpoint assay is easier to set up and automate than the plaque assay.

**SPECIAL CASE OF DEFECTIVE INTERFERING (DI) PARTICLES**

This chapter will conclude with a brief description of a special class of replicating virus particle referred to as a defective interfering (DI) particle. DI particles have been identified in most virus families. These “DI” particles are deemed defective because they cannot replicate autonomously, but instead require the presence of a helper virus to provide the function(s) that the defective particle lacks. The helper virus is usually the homologous virus from which the defective particle was derived. As their name implies, DI particles interfere with the replication of the helper virus and usually decrease the yield of the helper virus in mixed infections. Defective particles are assembled from the same structural proteins as their nondefective parent virus; however, the genomes of DI particles are defective and lack variable amounts of the normal genomic sequence. Although the genomes of defective particles are incomplete, they do retain the cis-acting sequences required for their replication and the sequences required for their encapsidation. DI particles derived from viruses with segmented genomes, such as influenza viruses and reoviruses, tend to have genomes in which one or more gene segment have significant deletions. Similarly, DI particles derived from viruses with a nonsegmented genome contain genomes with various degrees of deleted sequence. For example, DI particles of vesicular stomatitis virus may lack up to two-thirds of the normal genome. Morphologically, DI particles usually resemble the parental virions; however, with vesicular stomatitis virus, their normally bullet-shaped virions are shorter than wild-type virions. In the jargon used to
describe these particles, normal vesicular stomatitis virus
virions are called B particles and the DI particles are
called truncated or T particles.

The truncated genomes of DI particles are generated
through aberrant replication and/or recombination events
that lead variably to the mutation of gene sequences,
deletions, transpositions, duplications, and
even the insertion of gene sequences derived from host
DNA or RNA. Once generated, numbers of defective
particles increase greatly upon serial passage, particu-
larly when infections are performed at a high multiplic-
ty of infection. The rapid increase in defective particle
formation under these conditions is thought to result
from one or more of the following mechanisms: (1) their
shortened genomes require less time to be replicated,
thus over time the viral polymerase would replicate
more defective genomes than full-length genomes of the
helper virus; (2) the defective genomes are often tran-
scriptionally inactive, thus, they would be less often
diverted to serve as templates for transcription of
mRNA; (3) they may have enhanced affinity for the viral
replicase, giving them a competitive advantage over
their full-length counterparts. In essence, DI particles
appear to interfere with the replication of their helper
viruses by outcompeting the helper virus for critical
rate-limiting virus components such as the replicase
enzyme and/or structural proteins.

Our knowledge of DI particles derives mostly from
studies of viral infections of cultured cells. However, DI
particles are also generated by some viruses during
in vivo infections (eg, dengue, measles, hepatitis C, hep-
atitis A, and influenza A viruses), and evidence suggests
that they can interfere with replication of the helper virus
in vivo and alter the pathogenesis of the infection. This
phenomenon has been repeatedly demonstrated in experi-
mental animal model systems, but demonstrating a role
for DI particles in altering the course of natural infections
has been more difficult. Defective particles may alter the
pathogenesis of infection with the helper virus in vivo by
directly interfering with their replication (as described
above), and/or by stimulating antiviral innate immune
responses, such as the induction of type I interferon and
pro-inflammatory cytokines. Because of their ability to
interfere with virus replication and to alter the pathogene-
sis of infections with their parental virus, and in some
cases that of closely related heterologous viruses, DI par-
ticles have been studied for their potential use as antiviral
agents.

### TABLE 2.3 Data for Calculating TCID\textsubscript{50} Endpoints

| Virus Dilution | Mortality Ratio | Positive | Negative | Cumulative Positive | Cumulative Negative | Mortality Ratio | Percent Mortality |
|----------------|----------------|----------|----------|---------------------|---------------------|----------------|------------------|
| 10\textsuperscript{-3} | 8:8 | 8 | 0 | 23 | 0 | 23:23 | 100 |
| 10\textsuperscript{-4} | 8:8 | 8 | 0 | 15 | 0 | 15:15 | 100 |
| 10\textsuperscript{-5} | 6:8 | 6 | 2 | 7 | 2 | 7:9 | 78 |
| 10\textsuperscript{-6} | 1:8 | 1 | 7 | 1 | 9 | 1:10 | 10 |
| 10\textsuperscript{-7} | 0:8 | 0 | 8 | 0 | 17 | 0:17 | 0 |

For TCID\textsubscript{50} assays using microtiter plates, serial 10-fold dilutions of the virus sample are made in a cell culture medium. A sample volume (frequently 50 µL/well) of each dilution is added to several wells (the example above is 8 wells/dilution) of the microtiter plate. A suspension of indicator cells is then added to all wells of the culture plate. Plates are then incubated for a period of time that permits clear development of cytopathology for cytopathic viruses or until such time that viral growth can be detected by immunocytochemistry. Each well is scored as positive (dead) or negative (survive) for viral growth. For calculation by Reed–Muench, a cumulative “mortality” is tabulated and the percent mortality calculated. To calculate the 50% endpoint, the following formula is followed:

\[
\text{(% mortality at dilution next above 50\%) – 50\%} \\
\text{(% mortality at dilution next above 50\%) – (% mortality at dilution next below)}
\]

This gives the proportional distance between the dilutions spanning the 50% endpoint. For the data in this table, this gives:

\[
\frac{78 - 50}{78 - 10} - \frac{28}{68} = 0.41
\]

Adding this proportional factor to the dilution next above 50% (10\textsuperscript{-5}) yields a dilution of 10\textsuperscript{-5.4} to give one TCID\textsubscript{50}/50 µL (test volume). The reciprocal of this value, adjusting for the sample volume, gives the titer of the virus stock as: \(5 \times 10^6\text{TCID}_\text{50}/\text{mL}\).