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In the previous chapter, viruses were defined as obligate intracellular parasites that are unable to direct any 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 just a few proteins to others that encode more than 900 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 go through the same general steps for replication to occur—attach to a susceptible host cell, penetrate the cell, replicate its own genetic material and associated proteins, assemble new virus particles, and escape from the infected cell. This chapter will outline the general processes involved in each of these steps; however, more specific details are to be found in the chapters dealing with the individual virus families in Part II of this book.

GROWTH OF VIRUSES

Before the development of in-vitro cell culture techniques, all viruses had to be propagated in their natural host. For bacterial viruses, this was a relatively simple process that permitted an earlier development of laboratory-based research methods than was possible 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 experiment 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 agents affecting large animals. A most serious issue was the infection status of the recipient animals. An undetected infectious agent in a sheep, for example, 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, groups of animals were raised under more defined conditions for use in research studies. As new infectious agents were discovered and tests developed for their detection, the research animals became more “clean” and the concept of “specific pathogen-free” (SPF) animals was born. It is noteworthy, however, that animals apparently “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 viral and immunological studies were compromised by using rodents infected with mouse hepatitis virus, lactate dehydrogenase-elevating virus, or other agents unknown to the supplier of the research animals.

The search for suitable culture systems for 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 also known for fowlpox virus, a naturally occurring pathogen of birds. The use of embryonated chicken eggs then became routine for propagation efforts, not only for avian viruses, but also for viruses infecting mammalian species. Viruses in most of the animal virus families 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. 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 also provided a quantitative measure of the amounts of virus in individual stocks. Cell culture has largely replaced the egg system, which is labor-intensive and expensive, but the embryonated egg is still widely used for the isolation and growth of influenza viruses, in addition to many avian viruses.

The advent of in-vitro animal cell culture brought research studies in line with those involving bacterial viruses, thereby reducing the risks associated with adventitious viruses in animal inoculation systems and enhancing diagnostic testing. However, the problem of adventitious viruses was not entirely eliminated as, for example, early batches of the modified live poliovirus vaccine were contaminated with SV40 virus, a simian virus 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 viral contamination of the cell cultures used for virus isolation. In the veterinary world, contamination of ruminant cell cultures with bovine viral diarrhea virus has been an insidious and widespread problem. Some contaminated cell cultures and lines were probably derived from infected fetal bovine tissue, but—far more commonly—cells become 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 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.

Various in-vitro cell culture systems have been utilized since artificial medium was developed to maintain cell viability outside the source species: organ cultures, explant cultures, primary cell cultures, and cell lines. Organ cultures maintain the three-dimensional structure of the tissue and are utilized for short-term experiments. Tracheal epithelial cells that remain attached to the cartilage matrix of the trachea during culture are an example of this type of system. The creation of primary cell cultures utilizes proteases such as trypsin or collagenase to produce individual cells of a given tissue such as fetal kidney or lung, and the individual cells are permitted to attach to a cell culture matrix and will divide for a limited number of cell divisions. The limited lifespan of these cells requires continual production of the cells from new tissue sources, which can lead to variation in quality between batches. In contrast, theoretically, cell lines have an unlimited capability of division, so that a more standard viral growth system can be developed. In the early period of cell culture, the development of these immortalized cells (transformation) was an empirical process with a low probability of success. More recently, procedures have been developed to immortalize virtually any cell type, so that the number of cell lines representing different species is increasing rapidly.

**Recognition of Viral Growth in Culture**

Recognition of the presence of a viral agent in a host system was dependent initially upon the recognition of signs not found in an unaffected (control) host, death being the most extreme outcome but easiest to determine. The same essentially applies to cells in culture—detection of viral growth is dependent upon the detection of a property of the inoculated cells that is not found in control cultures maintained under identical growth conditions. As with animals, the sign of a viral infection of a culture that is easiest to detect is death of the cells or a very significant change in cell morphology. This is usually referred to as a cytopathic effect, or “CPE,” and is noted through microscopic examination of the test culture system (Figure 2.1). Careful observation of cultures showing cytopathic effect must be undertaken, as diagnostic samples may contain substances that are toxic for cultured cells, such as bacterial macromolecules. Other morphological changes may be manifest in virus-infected cell cultures; for example, a significant morphological change in cultured cells infected with avian reovirus is the formation of multinucleated cells or syncytia (Figure 2.1A). Many members of the family Paramyxoviridae can also 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 a distinct cytopathic effect of rounded cells, with or without small syncytia, which spreads very rapidly through a susceptible cell culture (Figure 2.1B).

In the search for unknown viruses in cell cultures, early researchers took advantage of the property of some viruses to bind to red blood cells. For example, cells infected with bovine parainfluenza virus 3 will show the ability to adsorb chicken red blood cells to the plasma membrane. In the budding process of virus maturation (orthomyxoviruses, paramyxoviruses), viral proteins are inserted into the plasma membrane. If these proteins bind to receptors on the red blood cells, the infected cells show adherence of the cells on their surface (hemadsorption) (Figure 2.1D). This property
of infected cells 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. The same viral proteins that permit hemadsorption are also responsible for the hemagglutination reaction. There are, however, viruses that can hemagglutinate red blood cells but not show hemadsorption of the infected cells—for example, adenoviruses and alphaviruses.

A characteristic morphological change in cells infected by certain viruses is the formation of inclusion bodies (Figure 2.2). These changes can be seen with a light microscope after fixation and treatment with cytological stains. As with hemadsorption, not all viruses will produce detectable inclusion bodies. The type of virus infecting a cell can be inferred by the location and shape of the inclusions: cells infected with herpesviruses, adenoviruses, and parvoviruses can have intranuclear inclusions, whereas cytoplasmic

**FIGURE 2.1** Cytopathic effects produced by different viruses. The cell monolayers are shown as they would normally be viewed by phase contrast microscopy, unfixed and unstained. (A) Avian reovirus in Vero cells. (B) Untyped herpesvirus in feline lung cell. (C) Bovine viral diarrhea virus in primary bovine kidney cells. (D) Parainfluenza virus 3 in Vero cells detected by hemadsorption of chicken red blood cells.

**FIGURE 2.2** Typical inclusions and abnormal cell morphology in virus-infected cells. (A) Reovirus inclusions (arrows) in infected Vero cells. (B) Canine distemper virus inclusions (arrows) and syncytium (arrowheads) in infected Vero cells. (C) Bovine adenovirus 5 intranuclear inclusions (arrows) in primary bovine kidney cells. (D) Transmission electron micrograph of an untyped adenovirus nuclear inclusion in A459 cells.
inclusions are characteristic of infections with poxviruses, orbiviruses, and paramyxoviruses (Figure 2.2B, 2.2C). The composition of the inclusions will vary with the virus type. The cytoplasmic Negri bodies identified in rabies-infected cells are composed of aggregates of nucleocapsids, whereas the intranuclear inclusions that occur in adenovirus-infected cells are crystalline arrays of mature virus particles (Figure 2.2D). Cytological stains are rarely used to identify cells infected with specific viruses, but are mainly used as a screening tests to assess the presence of any virus.

In the absence of a metagenomic screening test, detection of viruses that produce no cytopathology, do not induce hemadsorption or hemagglutinate, or produce no definable inclusions is carried out with virus-specific tests. This would be the case for screening bovine cells for the presence of non-cytopathic bovine viral diarrhea virus. The most commonly used tests in this type of situation are immunologically based assays such as fluorescent antibody tests or immunohistochemistry tests (Figure 2.3). The quality of the tests is dependent on the specificity of the antibodies used in the assay. With the development of monoclonal antibodies, this issue has been largely resolved. Other virus-specific tests depend on the detection of virus-specific nucleic acid in the infected cells. Hybridization tests have been replaced by assays based on polymerase chain reaction (PCR), because of their enhanced sensitivity and ease of test performance (see Chapter 5).

**VIRUS REPLICATION**

A fundamental characteristic that separates viruses from other replicating entities is the manner in which new virus particles are synthesized. Viruses do not use binary fission; virus particles are assembled *de novo* from the various structural components synthesized as somewhat independent but synchronized events. 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 non-attached 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 can be achieved with any type of virus (Figure 2.4). The

**FIGURE 2.3** Indirect fluorescent antibody detection of non-cytopathic bovine viral diarrhea virus (BVDV) infected cells. Bovine cells exposed to BVDV for 72 hrs were fixed with cold acetone. Fixed cells were stained with BVDV monoclonal antibody 20.10.6 followed by a goat anti-mouse serum tagged with fluorescein isothiocyanate.

**FIGURE 2.4** One-step growth curve of a non-enveloped 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 non-enveloped viruses are often released late and incompletely, when the cell lyses. The release of enveloped virions occurs concurrently with maturation by budding from the plasma membrane.
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. The period of time is referred to as the eclipse period, and represents the time needed for the various parts of the virus particle to be synthesized and assembled. Once assembly begins, there is an essentially exponential increase in infectious virus until the host cell is unable to maintain metabolic integrity. Depending on the type of virus, there may be sudden release of virus particles (lysis of the host cell, exemplified by T-even bacteriophage) or a more slow release (maturation of the virus particle at a cell membrane site, such as with influenza virus). A specific virus–host-cell system has its own inherent biological clock that cannot be significantly altered by a desire for faster test results, much to the frustration of the diagnostician, researcher, and clinician.

The one-step growth curve can be used to divide the virus replication cycle into its component parts for discussion of the general replication patterns. The basic components of the replication cycle are attachment, the eclipse period (penetration, uncoating, replication of component parts, maturation), and release of virus particles. Several of these patterns will be presented, but 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 binding of the virus particle to a host cell. This binding process may involve a series of interactions that define in part the host range of the virus and its tissue/organ specificity (tropism). Tissue and organ specificity largely defines the pathogenic potential of the virus and nature of the disease it induces. Virus particles interact with cell-surface molecules referred to as attachment factors, entry factors, receptors, and co-receptors. 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 specifically for viruses—rather, viruses have evolved to use host cell-surface molecules critical for cellular processes. Although the exact series of events that occur on the cell surface may be complex, a general process can be envisioned. Initial contact of a virus particle with the cell surface may involve short-distance electrostatic interactions with charged molecules such as heparan sulfate proteoglycans. That charge is a key factor in this initial interaction is supported by the observation that positively charged compounds such as diethyl amino ethanol (DEAE) dextran can increase binding of virus to host cells. This initial contact may be one that simply helps to concentrate virus on the surface of the cell, permitting a more specific interaction with other receptor-like molecules. The affinity of binding for a given site may be low, but the large number of potential binding sites makes the interactions nearly irreversible. Attachment of a virus particle to the host cell is a temperature-independent process, but penetration is dependent upon the fluidity of the lipids in the plasma membrane which does have temperature constraints.

In recent years, the search for receptors/entry factors mediating virus attachment and entry has intensified such that numerous candidate receptors/entry factors have been identified. These include ligand-binding receptors (e.g., chemokine receptors), signaling molecules (e.g., CD4), cell adhesion/signaling receptors [e.g., intercellular cell adhesion molecule-1 (ICAM-1)], enzymes, integrins, and glycoconjugates with various carbohydrate linkages, sialic acid being a common terminal residue (Table 2.1). The number

| 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                                                 |
| Coxsackievirus B               | Picornaviridae| CAR (coxsackie and adenovirus receptor)—Ig family                        |
| Human rhinovirus 14            | Picornaviridae| ICAM-1 (intercellular cell adhesion molecule-1)—Ig family               |
| Echovirus 1                    | Picornaviridae| α2β1 integrin VLA-2                                                     |

(Continued)
of specific 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. Different viruses may use the same receptor/entry factor, which simply reflects the fact that a similar host cell serves as the replication site of the viruses. The process of identification of 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; for foot-and-mouth disease virus, the receptors in the bovine host are integrins, but cell-culture passaged virus can use heparan sulfate. This change in receptor specificity alters the pathogenicity of the virus, clearly indicating that receptor specificity is a key factor in the disease process. For viruses with a wide host range, such as some of the alphaherpesviruses, it is speculated that these viruses can use several receptors, accounting for their ability to grow in cells from many hosts.

The story of virus–receptor interaction is further complicated by those viruses that require several entry factors to initiate an infection successfully. A prominent example of this phenomenon is human immunodeficiency virus (HIV). Initial cell interaction is through heparan sulfate, followed by binding to the CD4 receptor and a chemokine receptor such as CXCR4 or CCR5. For hepatitis C, at least four entry factors (CD81, SR-BI, CLDN1, and OCLN) must be expressed on the cell surface before the cell becomes fully susceptible to infection. It may well be that the highly restricted host range of some viruses is reflective of unique entry factor

| Virus                          | Family             | Receptor                                      |
|-------------------------------|--------------------|-----------------------------------------------|
| Foot-and-mouth disease virus—wild-type virus | Picornaviridae    | Various integrins                             |
| Foot-and-mouth disease virus—cell-culture-adapted | Picornaviridae    | Heparan sulfate proteoglycan                  |
| Feline calicivirus            | Caliciviridae      | fJAM-A (feline junction adhesion molecule-A)  |
| Adenovirus 2                  | Adenoviridae       | CAR-Ig family                                 |
| Adenoviruses                  | Adenoviridae       | αv33, αv35 integrins                          |
| Herpes simplex virus 1        | Herpesviridae      | HveA (herpes virus entry mediator A), 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        | TIR-1 (transferrin receptor-1)                |
| Adenovirus-associated virus 5 | Paroviridae        | α(2,3)-linked sialic acid                     |
| Influenza A virus             | Orthomyxoviridae   | Sialic acid                                   |
| Influenza C virus             | Orthomyxoviridae   | 9-O-acetylsialic acid                         |
| Canine distemper virus        | Paramyxoviridae    | SLAM (signaling lymphocyte activation molecule) |
| Newcastle disease virus       | Paramyxoviridae    | Sialic acid                                   |
| Bovine respiratory syncytial virus | Paramyxoviridae   | Unknown                                       |
| Hendra virus                  | Paramyxoviridae    | Ephrin-B2                                     |
| Rotavirus                     | Reoviridae         | Various integrins                             |
| Reovirus                      | Reoviridae         | JAMs (junction adhesion molecules)            |
| Mouse hepatitis virus         | Coronaviridae      | CEA (carcinoembryonic antigen)—Ig family      |
| Transmissible gastroenteritis virus | Coronaviridae    | Aminopeptidase N                              |
| Lymphocytic choriomeningitis virus | Arenaviridae   | α-Dystroglycan                                 |
| Dengue virus                  | Flaviviridae       | Heparan sulfate proteoglycan                  |
| Rabies virus                  | Rhabdoviridae      | Acetylcholine, NCAM (neural adhesion molecule) |
requirements found only on highly differentiated cells. A somewhat indirect entry factor system is exemplified by dengue virus. Virus particles bound to so-called non-neutralizing antibodies gain entry to macrophages by virtue of the Fc receptor binding to the immunoglobulin molecule. The antibody–virus complex is internalized, with infectious virus being released into the cytoplasm of the phagocytic cell. Foot-and-mouth disease virus and feline coronavirus can also use this antibody-dependent enhancement entry system \textit{in vitro}, but its importance in the natural infection process is conjectural.

**Penetration**

The function of the receptors/entry factors is not simply to bind the virus particle, but also to assist or direct the process whereby the virus enters the cell. This assistance can occur through several different processes: (1) the interaction of the virus with its receptor can result in a conformational change in the virus particle or attachment protein that is necessary for entry; (2) the concentration and/or immobilization of the cell receptor can trigger a cellular response leading to internalization of the complexed receptors; (3) the bound receptor may induce the movement of the complex to an appropriate entry site or to a site permitting contact with a co-receptor. The individual cellular receptor utilized by a specific virus can determine the mode of entry of the virus into the host cell. All the signaling processes used by the host cell to internalize macromolecules and receptors are used in the virus entry process, as viruses rely on the normal cellular processes—specifically, activation of a myriad of protein kinases, GTPases, binding proteins, second messengers, and structural element rearrangements. The specific pathways activated depend on the type of entry process. For virus particles, the internalization process can be divided into two general patterns: entry by way of membrane-bound vesicles or direct entry at the plasma membrane. Viruses that utilize the membrane vesicle route must still pass through a limiting membrane to gain access to the cytosol, so that the manner in which viruses move across the limiting membrane may be similar regardless of the site at which this event takes place.

The mechanism whereby viruses are internalized in membrane-bound vesicles is generally designated as endocytosis, a normal cellular process for internalizing macromolecules. The earliest example of this process involved the identification of virus particles in clathrin-coated “pits” in the plasma membrane (Figure 2.5). The cellular protein dynamin-2 induces the formation of a vesicle that enters the early endosomal system. The virus particle in the early endosome is subjected to a drop in pH that may induce structural changes in the particle. In the classic pathway, the vesicle is transformed into a late endosome with a corresponding lower pH. For some viruses, it is this lower pH that induces the structural changes. The late endosome will then fuse with lysosomal vesicles to initiate the degradation of the material internalized by this process. Virus particles that do not successfully escape the endosome are degraded and prevented from initiating the infection process. The one noted exception to this is reovirus that uses the lysosomal enzymes to activate the penetration process. The key factors in this entry process are the changes
in pH which are necessary to induce structural changes in the virus particles that permit the breaching of the limiting membrane boundary. Compounds (bafilomycin A1, chloroquine, NH₄Cl) that can prevent the lowering of the pH in endosomes can significantly impair the infection process.

A second major vesicle internalization process involves the caveosome system. Caveolin-coated pits and lipid-raft-mediated pits containing cholesterol with associated virus particles are internalized and enter the caveosome system. Unlike the endosomal system, the caveosomes maintain a neutral pH within the vesicle, but there appears to be a pathway for caveosomes to enter the endosomal system, allowing pH activation of the virus particles. Another route for the caveosomes is to the endoplasmic reticulum, where viruses such as SV40 cross the limiting membrane boundary. As a general rule, the 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. The boundary between these systems may not be as clearly defined as was initially believed, and some viruses may use different systems depending on the cell type encountered. Earlier studies on virus particle entry mechanisms may be compromised by the possibility that these observations included predominantly non-infectious particles that were processed differently than infectious particles.

The true entry of the viral genetic material into the host cells occurs when the genome enters the cytosol. For the enveloped viruses, this process is conceptually simple, in that the viral envelope need only fuse with the cellular membrane, be it at the cell surface or within an endosomal vesicle. For many of the viruses in the family Paramyxoviridae, the fusion process occurs at the plasma membrane under neutral pH conditions. This group of viruses can induce a phenomenon referred to as “fusion from without” whereby, at high multiplicity of infection and in the absence of virus replication, the plasma membranes of adjacent cells fuse to form multinucleated cells known as syncyta. For many viruses, the fusion process is a pH-activated one, requiring the virus particles to be in the endosomal pathway. In all cases, regardless of the pH requirements, the surface glycoproteins of the virus particles must undergo changes in their tertiary or quaternary structures such that generally hydrophobic regions of the proteins can come into contact with the cellular membrane to produce a localized destabilization and induce fusion with the viral envelope. For many of the viruses (paramyxoviruses, orthomyxoviruses, flaviviruses, coronaviruses, and alphaviruses, for example), the surface glycoproteins must undergo a proteolytic processing step in order to permit these necessary conformational changes to occur. This finding was a major discovery in the early 1970s that allowed the routine propagation of influenza virus and parainfluenza viruses in cell culture.

The breaching of cellular membranes by non-enveloped virus particles is conceptually more difficult and potentially more confusing than the membrane fusion process. Several different mechanisms have been identified (Figure 2.6). For the picornaviruses, a “pore mechanism” has been proposed that operates under neutral or acid pH, depending on the specific virus. For poliovirus, the key event in penetration is the structural rearrangement of the virus particle induced by the binding of the cellular receptor. In the restructuring process, VP4 is released and the myristylated N-terminus of the VP-1 protein is inserted into the plasma membrane. The viral RNA then enters the cytoplasm through a pore in the membrane. In this model, the penetration and uncoating steps of the replication cycle occur simultaneously, with the viral capsid proteins remaining at the plasma membrane. For adenoviruses, a complex set of reactions occurs in the low pH of the endosomes, the result of which is the dissolution of the cellular membrane (lytic mechanism) such that a modified virus particle enters the cytoplasm along with the contents of the endosome. For polyomaviruses, the virus particle is transported to the endoplasmic reticulum by the caveosome system. In conjunction with cellular chaperone proteins, the virus particle is transported across the membrane (transfer mechanism) into the cytoplasm. In all cases, the virus particles are modified by rearrangements induced by receptor binding, pH, protease cleavages, or binding by cellular transport proteins.

The entry of the virus particle, nucleocapsid, or genomic nucleic acid into the cytoplasm in many cases is not the final step in the initiation of the replication process for the virus. In most cases, the nucleic acid is not in a form that would permit the synthesis of plus-strand RNA needed to direct the production of viral proteins, and the genome is not in a correct location for replication to occur. Again, cellular processes are involved in the transport of the viral units to the required locations. For most of the longer translocation needs, the microtubule transport system is used; actin filaments enable 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 (parvoviruses) or inducing the transport of the nucleic acid into the nucleus (adenoviruses, herpesviruses). The environment of the cytoplasm or the nucleus completes the uncoating process by inducing conformational changes in the nucleocapsid proteins by binding to “replication” sites, by allowing further proteolytic processing of proteins, or by destabilizing the viral protein–nucleic acid interactions. As an example, Semliki Forest virus capsid proteins, after release from the endosomes, bind to 60S ribosomal subunits that permit
the release of the viral RNA. For picornaviruses, the entry process produces a genomic RNA that is capable of interacting directly with ribosomes to initiate viral protein synthesis. Table 2.2 provides some general characteristics of the replication cycle of the families of viruses that 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 has been somewhat passive in that, for the most part, no biosynthetic activity directed by the viral nucleic acid has occurred. The preliminary steps have put the viral genome in position to take active control of its replication cycle and to remodel the cell to assist in the production of mature virus particles.

The details of the next phases of the replication cycle are unique to each of the virus families, and those are outlined in each of the chapters on the specific virus families in Part II. A few selected examples of different replication strategies will be described in succeeding pages to emphasize specific aspects of their replication cycles.

Representative Examples of Virus Replication Strategies

**Picornaviruses**

For the picornaviruses, the entry process—whether at the plasma membrane or within an endosomal vesicle—results in an uncoated positive-sense single-stranded RNA genomic
molecule free in the cytoplasm (Figure 2.7). In this environment, there are no cellular polymerases that are capable of replicating the genomic RNA. Accordingly, the first major event for the genomic RNA is to associate with the cellular protein translational system. Unlike most host-cell messenger RNAs (mRNAs), the picornavirus genomic RNA lacks a standard 5' cap structure. However, these viruses have evolved to be able to initiate protein synthesis using an internal ribosome entry site (IRES). This alternative mechanism for binding ribosomes and translation factors provides the virus with the ability to restrict host-cell mRNA translation by directing the cleavage of translation initiation factors necessary for cap-dependent translation. This inhibition of cellular translation reduces competition for ribosomal complexes and

### Table 2.2 Characteristics of Replication of Viruses of Different Families

| Family         | Uptake Route                          | Site of Nucleic Acid Replication | Site of Maturation (Budding) |
|----------------|---------------------------------------|---------------------------------|-----------------------------|
| Poxviridae     | Variable                              | Cytoplasm                       | Cytoplasm                   |
| Astaviridae    | Clathrin-mediated endocytosis         | Cytoplasm                       | (Plasma membrane)           |
| Iridoviridae   | Variable                              | Nucleus/cytoplasm               | Cytoplasm                   |
| Herpesviridae  | Variable                              | Nucleus                         | (Nuclear membrane)          |
| Adenoviridae   | Clathrin-mediated endocytosis         | Nucleus                         | Nucleus                     |
| Polyomaviridae | Caveolar endocytosis                  | Nucleus                         | Nucleus                     |
| Papillomavirida| Clathrin/caveolar endocytosis         | Nucleus                         | Nucleus                     |
| Parvoviridae   | Clathrin-mediated endocytosis         | Nucleus/cytoplasm               | Nucleus                     |
| Hepadnavirida  | Clathrin-mediated endocytosis         | Nucleus/cytoplasm               | (Endoplasmic reticulum)     |
| Retroviridae   | Plasma membrane fusion or clathrin-mediated endocytosis | Nucleus | (Plasma membrane)       |
| Reoviridae     | Clathrin-mediated endocytosis         | Cytoplasm                       | Cytoplasm                   |
| Paramyxovirida | Plasma membrane fusion                | Cytoplasm                       | (Plasma membrane)           |
| Rhabdovirida   | Plasma membrane fusion                | Cytoplasm                       | (Plasma membrane)           |
| Filoviridae    | Plasma membrane fusion                | Cytoplasm                       | (Plasma membrane)           |
| Bornaviridae   | Clathrin-mediated endocytosis         | Nucleus                         | (Plasma membrane)           |
| Orthomyxovirida| Clathrin-mediated endocytosis         | Nucleus/cytoplasm               | Nucleus                     |
| Bunyaviridae   | Clathrin-mediated endocytosis         | Cytoplasm                       | (Golgi membrane)            |
| Arenaviridae   | Clathrin-mediated endocytosis         | Cytoplasm                       | (Plasma membrane)           |
| Coronaviridae  | Clathrin-mediated endocytosis/plasma membrane fusion | Cytoplasm | (Endoplasmic reticulum) |
| Arteriviridae  | Clathrin-mediated endocytosis         | Cytoplasm                       | (Endoplasmic reticulum)     |
| Picornaviridae | Caveolar endocytosis/plasma membrane insertion | Cytoplasm | Cytoplasm                 |
| Caliciviridae  | Caveolar endocytosis/plasma membrane insertion? | Cytoplasm | Cytoplasm                 |
| Astroviridae   | Caveolar endocytosis/plasma membrane insertion? | Cytoplasm | Cytoplasm                 |
| Togaviridae    | Clathrin-mediated endocytosis         | Cytoplasm                       | (Plasma membrane)           |
| Flaviviridae   | Clathrin-mediated endocytosis         | Cytoplasm                       | (Endoplasmic reticulum)     |
reduces the ability of the cells to produce an array of antiviral molecules that are made in response to the viral infection (see Chapter 4). The picornavirus proteins are synthesized from a single long open reading frame (ORF), with generation of specific proteins by a series of proteolytic cleavages that are mainly directed by virus-encoded proteases. It is one of these proteases that cleaves the cellular translation factor eIF4G.

A limited amount of viral protein can continue to be made from the input genome; however, the virus must begin to direct the synthesis of viral RNA if the infection is to be a productive one. As cellular enzymes are incapable of doing this, a key viral protein is an RNA-dependent RNA polymerase—3Dpol for the picornaviruses. This protein, produced by a series of proteolytic cleavage reactions from precursor protein VP3, is found associated with remodeled smooth cellular membranes and this unit is referred to as the “RNA replication complex.” Protein 3Dpol is a primer-dependent enzyme that also requires the presence of at least six other viral proteins for all aspects of RNA synthesis to be completed—synthesis of negative-stranded RNA as a template for the
positive strand and more positive-stranded RNA to direct protein synthesis and for packaging into the virus particles. The mature virus particle of picornaviruses is composed of three or four proteins proteolytically processed by a virus-encoded protease from the precursor protein, VP1. The capsid proteins assemble into a 60-subunit particle with a single copy of the genomic RNA. The exact mechanism for the insertion of the RNA into the developing capsid remains unclear. The rate-limiting process for particle maturation appears to be the availability of the RNA molecule containing the VPg primer protein at the 5’ end. Picornaviruses do not have a specific mode of exit from the infected cell, as crystalline arrays of virus occur in the cytoplasm of infected cells awaiting dissolution of the cell structure.

The replication pattern of picornaviruses illustrates several common elements that are also found in other virus replication systems. Entry of the virus particle can occur at the plasma membrane or in membrane-bound vesicles. The first task of the positive-sense genomic RNA is to associate with ribosomes to produce new viral proteins that begin the process of replicating the viral RNA and taking control of the host-cell metabolic process. Viral proteins can be synthesized as larger precursor proteins that are cleaved by virus-specific proteases. They can induce the remolding of cellular membrane structures to provide sites for viral RNA synthesis. Viral proteases perform several functions—generation of mature viral proteins and cleavage of various host-cell proteins that directly affect protein and RNA synthesis, in addition to inhibiting transcription of new proteins in response to the infection.

**Rhabdoviruses**

The rhabdoviruses present a different mode of replication (compared with picornaviruses) in that the input genomic RNA is of a negative sense—that is, it cannot serve as an mRNA for protein synthesis (Figure 2.8). The infection process is initiated by attachment of the virus particle to the plasma membrane and entry into an endosomal vesicle. The virion glycoproteins induce the fusion of the viral envelope with the endosomal membrane, with the release of the helical nucleocapsid into the cytoplasm. Unlike the picornaviruses, the virion RNA of rhabdoviruses is complexed throughout its length with N protein. Also unlike picornaviruses, the first event for the rhabdoviruses is to transcribe the virion RNA to make mRNA molecules. To do this, the nucleocapsid must contain an RNA transcriptase, because there are no cellular cytoplasmic enzymes that can fulfill this role. A series of capped, polyadenylated monocistronic RNAs are generated beginning at the 3’ end of the genomic RNA. This is achieved through a series of start and stop signals in the genomic RNA, and results in graded amounts of product from the 3’ to the 5’ end. As protein synthesis progresses, a complete plus strand (antigenome) of viral RNA must be made to begin the process of producing more negative-sense virion RNA. The signal for this switch from discrete mRNA molecules to a complete genomic sequence appears to be the complexing of the RNA with N protein. Newly synthesized virion RNA can serve as template for more mRNA (secondary transcription) or can be incorporated into nucleocapsids for transport to a membrane maturation site.

Maturation of rhabdoviruses occurs through a series of coordinated movements of viral components. The virion glycoproteins are synthesized in association with the rough endoplasmic reticulum and transported to the surface of the cells through the exocytotic pathway. A key protein in the maturation process is the matrix (M) protein. This protein can bind to the nucleocapsid as well as to modified areas of the inner surface of the plasma membrane. These modified areas or “lipid rafts” have been identified as maturation sites for several of the virus families that contain membranes. It appears that the cytoplasmic tails of the surface glycoproteins are also critical for efficient budding of virus particles, but the exact signals that are necessary for the modified plasma membrane to produce the mature virion remain unknown. Unlike the picornaviruses, in the rhabdoviruses there are no mature virus particles in the infected cell. The maturation of the virus particles at the plasma membrane provides the mechanism for egress from the infected cell.

Like the picornaviruses, rhabdovirus activity is aimed towards specific inhibition of the host-cell innate antiviral defense systems described in Chapter 4. However, with a capped message system, modification of the translation process is not an option. In addition, viral proteases are not involved in the replication process, so direct degradation of host proteins is also not utilized. The paramyxoviruses and rhabdoviruses exert their inhibitory activity primarily on the interferon system—both the stimulation of interferon synthesis and the induction of an antiviral state by interferon. This appears to be achieved by competitive binding to internal pathogen recognition receptors (sensor-like elements such as MDA5 and RIG-I) that initiate the pathway for induction of interferon synthesis, or by preventing the transcription of interferon response genes (ISGs) by binding of viral proteins to the activators of transcription, resulting in their degradation. Inhibition of the induction of interferon is likely to be important during infection of animals, wherein interferon produced in one cell exerts its antiviral effect on its neighbors.

**Retroviruses**

The family **Retroviridae** provides several unique elements not found among viruses in the picorna- and rhabdovirus families (Figure 2.9). Penetration of the host cell is similar to that by the paramyxoviruses, in that most viruses appear
Figure 2.8 Single-cell reproductive cycle of a rhabdovirus. The virion binds to a cellular receptor and enters the cell via receptor-mediated endocytosis (1). The viral membrane fuses with the membrane of the endosome, releasing the helical viral nucleocapsid (2). This structure comprises (−) strand RNA coated with nucleocapsid protein molecules and a small number of L and P protein molecules, which catalyze viral RNA synthesis. The (−) strand RNA is copied into five subgenomic mRNAs by the L and P proteins (3). The N, P, M, and L mRNAs are translated by free cytoplasmic ribosomes (4), while G mRNA 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 full-length (+) strand copy of genomic RNA, 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 RNA in the form of nucleocapsids (7). Some of these newly synthesized (−) strand RNA molecules enter the pathway for viral mRNA synthesis (8). Upon translation of G mRNA, the G protein enters the secretory pathway (9), in which it becomes glycosylated and travels to the plasma membrane (10). Progeny nucleocapsids and the M protein are transported to the plasma membrane (11 and 12), where association with regions containing the G protein initiates assembly and budding of progeny virions (13). [From Principles of Virology, S. J. Flint, L. W. Enquist, V. R. Racaniello, A. M. Skalka, 3rd ed., vol. 1, p. 534. Copyright © Wiley (2008), with permission.]
FIGURE 2.9  Single-cell reproductive 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 identities of receptors are known for several retroviruses. The viral core is deposited into the cytoplasm (2) following fusion of the virion and cell envelopes. Entry of some beta- and gammaretroviruses may involve endocytic pathways. The viral RNA genome is reverse transcribed by the virion reverse transcriptase (RT) (3) within a subviral particle. The product is a linear double-stranded viral DNA with ends that are shown juxtaposed in preparation for integration. Viral DNA and integrase (IN) protein 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 site-specific insertion of the viral DNA ends, which can take place at virtually any location in the host genome (5). Transcription of integrated viral DNA (the provirus) by host cell RNA polymerase II (6) produces full-length RNA transcripts, which are used for multiple purposes. Some full-length RNA molecules 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 RNA molecules are destined to become encapsidated as progeny viral genomes (9). Other full-length RNA molecules are spliced within the nucleus (10) to form mRNA for the Env polyprotein precursor proteins. Env mRNA is translated by ribosomes bound to the endoplasmic reticulum (ER) (11). The Env proteins are transported through the Golgi apparatus (12), where they are glycosylated and cleaved by cellular enzymes to form the mature SU-TM complex. Mature envelope proteins are delivered to the surface of the infected cell (13). Virion components (viral RNA, Gag and Gag-Pol precursors, and SU-TM) assemble at budding sites (14) with the help of cis-acting signals encoded in each that exploit intracellular vesicular trafficking machinery. Type C retroviruses (e.g., 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 the core precursor polyprotein. During or shortly after budding, PR cleaves at specific sites within the Gag and Gag-Pol precursors (16) to produce the mature virion proteins. This process causes a characteristic condensation of the virion cores. [From Principles of Virology, S. J. Flint, L. W. Enquist, V. R. Racaniello, A. M. Skalka, 3rd ed., vol. 1, p. 531. Copyright © Wiley (2008), with permission.]
to enter under neutral pH conditions at the plasma membrane. Specialized areas of the membrane may be selected through lateral movement of the virus–receptor complex. Structural rearrangements occur between the membrane proteins SU and TM during the entry process; the TM or transmembrane protein is believed to play the major role in membrane fusion. The nucleocapsid enters the cytoplasm and moves on cytoskeletal fibers to specified replication sites. The events in the uncoating process in the cytoplasm are poorly understood, but the genomic RNA becomes accessible to the RNA-dependent DNA polymerase found in the virion, as DNA synthesis is the first metabolic event in the retrovirus life cycle. Unlike picornaviruses that initially produce viral proteins, or rhabdoviruses that first produce mRNA, retroviruses use their unique polymerase to transcribe the plus-strand RNA into a double-stranded DNA copy. The virion RNA (two copies, making the virus essentially diploid) is capable of translation, but this is not the fate of the incoming genomic RNA.

A double-stranded linear DNA molecule is produced through a series of complex reactions, beginning with the priming of DNA using primer transfers RNAs (tRNAs) incorporated in the mature virion. The linear DNA molecule, still associated with a modified capsid structure, must be transported to the nucleus for integration into the host-cell genome. For many of the retroviruses, this transport and integration step is linked to cell division and the viral DNA is not available to integrate into host DNA until the nuclear membrane dissociates. The exceptions to this rule are the lentiviruses and spumaviruses, which can integrate their DNA into non-dividing cells. The integration of the linear DNA into the host genome is directed by the viral integrase enzyme. It is from the integrated DNA that new viral RNA is synthesized. This process is carried out by the cellular transcription systems and the resulting RNA has all the properties of mRNA, such as a 5’ cap and a poly(A) tail at the 3’ end. A unique feature of the retroviruses is that viral messages may be spliced, thus joining two discontinuous pieces of RNA into a single virus-encoded message. This method of producing viral mRNA is not found with the picorna- or rhabdoviruses. The viral RNA molecules are transported to the cytoplasm as normal cellular mRNA, where they either associate with ribosomes for the synthesis of viral proteins or become incorporated into nucleocapsid structures.

As retroviruses are enveloped entities, the maturation process is similar but different from that of rhabdoviruses. The Env precursor protein is proteolytically processed during its synthesis in the endoplasmic reticulum and Golgi, and is transported to the cell surface as the TM and SU envelope proteins (C-type virions). A unique feature of the retroviruses is the manner in which the precursor proteins Gag and Gag-Pro-Pol participate in the maturation process. For many viruses, it is the mature proteins that are incorporated into the virion. For the retroviruses, the Gag protein enters the process as the precursor to matrix, capsid, and nucleocapsid proteins. A portion of the uncleaved precursor functions in a similar manner as the matrix protein of the paramyxoviruses and rhabdoviruses, providing a possible link with the cytoplasmic tail of the envelope protein and also binding to the membrane lipids. The presence of homologous viral glycoproteins is not essential for budding, as “bald” particles can be produced in addition to pseudotypes—virus particles with unrelated viral surface proteins. Another portion of Gag interacts with the virion RNA and may be responsible for the packaging of the RNA into the developing virion. As the Gag precursor associates with the inner surface of the plasma membrane, a budding site is established for the production of immature virus particles. Maturation for most retroviruses takes place once the virus particle is released from the plasma membrane. It is at this time that a viral protease cleaves the precursor proteins to produce the mature infectious particle. Inhibition of the protease produces non-infectious particles. As with picornaviruses, a virus-encoded protease has a key role in the replication process, but the stage in the replication process is very different for the retroviruses.

**Adenoviruses**

The adenoviruses are the last virus family to be described in this section, and for information on other families the reader is referred to Part II of this book. Adenoviruses are non-enveloped DNA viruses, approximately 90 nm in diameter with fibers projecting from the vertices of the icosahedron. The initial interaction with a host cell is through the fiber proteins and the coxsackievirus B and adenovirus receptor (CAR) receptor, which is the receptor for most human adenoviruses (Figure 2.10). This high-affinity binding brings the penton base protein into contact with cellular integrins, which are cell-surface receptors for extracellular matrix proteins. This binding initiates the endocytotic process involving clathrin-coated pits. Under the influence of the low pH of the endosome, the capsid structure of the virion undergoes modifications, with the loss of several virion proteins and the possible cleavage of others by the virion-associated protease. A “lytic” reaction releases the modified virion into the cytoplasm (Figure 2.6). After release from the endosome, the virions associate with the microtubule system for transport to the nucleus. At the nuclear membrane, the virion associates with the nuclear pore complex. Under the influence of the nuclear pore complex and histones, the viral DNA separates from the major virion proteins and is transported into the nucleus.

For large DNA viruses such as adenoviruses, viral gene expression can be divided into two major blocks, early and late, but these divisions have exceptions. Transcription of the viral DNA utilizes the host-cell transcription system, including the generation of spliced mRNAs. The early
FIGURE 2.10 Single-cell reproductive cycle of human adenovirus type 2. The virus attaches to a permissive human cell via interaction between the fiber and (with most serotypes) the coxsackie-adenovirus receptor on the cell surface. The virus enters the cell via endocytosis (1 and 2), a step that depends on the interaction of a second virion protein, penton base, with a cellular integrin protein (red cylinder). Partial disassembly takes place prior to entry of particles 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). Following alternative splicing and export of E1A mRNAs to the cytoplasm (6), E1A proteins are synthesized by the cellular translation machinery (7). These proteins are imported into the nucleus (8), where they regulate transcription of both cellular and viral genes. 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). Some late promoters are activated simply by viral DNA replication, but maximally efficient transcription of the major late transcription unit (Fig. 1, ML) requires the late IVa2 and L4 proteins. Processed late mRNA species are selectively exported from the nucleus as a result of the action of the E1B 55-kDa and E4 Orf6 proteins (16). Their efficient translation in the cytoplasm (17) requires the major VA RNA: VA RNA-I, which counteracts a cellular defense mechanism, and the late L4 100-kDa protein. 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 non-infectious 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 Principles of Virology, S. J. Flint, L. W. Enquist, V. R. Racaniello, A. M. Skalka, 3rd ed., vol. 1, p. 504. Copyright © Wiley (2008), with permission.]
genes have at least three functions: (1) production of proteins needed for DNA replication; (2) establishment of systems for the inhibition of host-cell defenses; (3) stimulation of the cell to undergo cell division, which enhances virus replication. The first protein to be expressed is the large E1A protein, which has several effects on transcription units of other adenoviral proteins. With the onset of DNA synthesis, a large set of late genes are expressed, many from spliced mRNAs. The package of late genes includes the structural proteins of the mature virions. Structural proteins are synthesized in the cytoplasm and transported to the nucleus for assembly. Unlike the picornaviruses with 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 co-factor to prevent premature proteolysis completes the maturation process by degrading scaffold proteins and cleaving precursor proteins. As with picornaviruses, adenovirus-infected cells show large crystalline arrays of virus particles but, in this instance, these are found as large intranuclear inclusion bodies.

The complexity of the adenovirus replication cycle and the long period (10–24 hours) to complete the cycle requires that the virus effectively inhibits the various antiviral responses available to the acutely infected cell. Adenoviruses effectively inhibit the cellular responses in many different ways. An early expressed protein of adenoviruses is the E1A protein, which can induce non-dividing cells to enter the S phase. This growth stimulation can be viewed by the cell as an abnormal event, with the induction of apoptosis (programmed cell death, see Chapter 4) that would not benefit the virus, so adenoviruses in turn produce several proteins (including E1B-19K) to block the induction of apoptosis. As with the picorna- and paramyxoviruses, adenoviruses also are capable of specifically suppressing both the production of interferon and the interferon response pathways. Part of this inhibition is mediated by small RNAs that inhibit the protein kinase (PKR) pathway that is important in interferon-mediated antiviral resistance (see Chapter 4). Viral RNAs can also interfere with the action of the interfering RNAs (RNAi) that the cell can use to inhibit the translation of viral mRNAs. Finally, adenoviruses can inhibit the synthesis of host-cell proteins by preventing the export of cellular mRNAs from the nucleus and by blocking translation of host-cell messages through modifications of the translation initiation factors. As with all viral infections, adenoviruses have evolved innovative strategies to circumvent the host-cell processes that limit virus replication and the production of new virus particles.

Assembly and Release

In the four examples just presented, two of the virus families were non-enveloped viruses and two were enveloped. All non-enveloped animal viruses have an icosahedral structure with varying degrees of complexity. The structural proteins of simple icosahedral viruses such as parvovirus and picornaviruses can associate spontaneously to form capsomers, which self-assemble to form capsids into which viral nucleic acid is packaged. Completion of the virion assembly often involves proteolytic cleavage of one or more species of capsid protein. Most non-enveloped viruses accumulate within the cytoplasm or nucleus and are released only when the cell eventually lyses. As indicated with adenovirus, the simple “self-assembly” model does not hold for the larger icosahedral viruses, as virus-encoded scaffolding proteins are needed to bring the capsids proteins into correct alignment to form a functional virus particle. Mutations in either the scaffolding proteins or the proteases that degrade these structures generate lethal mutations with respect to the production of infectious virus particles. These virus-encoded proteases can be targets for the development of antiviral agents.

All mammalian viruses with helical nucleocapsids, in addition to some with icosahedral nucleocapsids (e.g., herpesviruses, togaviruses, and retroviruses), mature by acquiring an envelope as they bud through cellular membranes. Enveloped viruses bud from the plasma membrane, from internal cytoplasmic membranes, or from the nuclear membrane. Viruses that acquire their envelope within the cell are then transported in exocytotic vesicles to the cell surface. Insertion of the viral glycoprotein(s) into the lipid bilayer of membranes occurs with lateral displacement of cellular proteins from that patch of membrane (Figure 2.8). Monomeric viral glycoprotein molecules associate into oligomers [homotrimers for the hemagglutination protein (HA) of influenza virus] to form the typical rod-shaped spike (peplomer) or club-shaped peplomer with a hydrophilic domain projecting from the external surface of the membrane, a hydrophobic transmembrane anchor domain, and a short hydrophilic cytoplasmic domain projecting slightly into the cytoplasm. In the case of icosahedral viruses (e.g., togaviruses), each protein molecule of the nucleocapsid binds directly to the cytoplasmic domain of the membrane glycoprotein oligomer, thus molding the envelope around the nucleocapsid. In the more usual case of viruses with helical nucleocapsids, it is the matrix protein that binds to the cytoplasmic domain of the glycoprotein spike (peplomer); in turn the nucleocapsid protein recognizes the matrix protein and this initiates budding of infectious virus particles. Release of each enveloped virion does not disrupt the integrity of the plasma membrane, hence thousands of virus particles may be shed over a period of several hours or days without significant cell damage.
Epithelial cells display polarity—that is, they have an apical surface facing the outside world and a basolateral surface facing the interior of the body; the two are separated by lateral cell–cell tight junctions. These surfaces are chemically and physiologically distinct. Viruses that are shed to the exterior (e.g., influenza virus) tend to bud from the apical plasma membrane, whereas others (e.g., C-type retroviruses) bud through the basolateral membrane and are free to proceed to other sites in the body, sometimes entering the bloodstream and establishing systemic infection (Figure 2.11).

Flaviviruses, coronaviruses, arteriviruses, and bunyaviruses mature by budding through membranes of the Golgi complex or rough endoplasmic reticulum; vesicles containing the virus then migrate to the plasma membrane with which they fuse, thereby releasing the virions by the process of exocytosis (Figure 2.12). Uniquely, the envelope of the herpesviruses is acquired by budding through the inner lamella of the nuclear membrane; the enveloped virions then pass directly from the space between the two lamellae of the nuclear membrane to the exterior of the cell via the cisternae of the endoplasmic reticulum.

The budding process for some viruses may not be the final step in release of an infectious virus particle. As was noted for retroviruses, the Gag protein complex within the virus must be proteolytically processed to produce an infectious virus particle. For influenza virus, the virus must escape from the surface structures of the host cell. For this the virus needs to have an active neuraminidase enzyme to cleave the sialic acid residues from the macromolecules on the cell surface. Without the neuraminidase activity, the emerging virus particle becomes trapped at the cell surface.
QUANTITATIVE ASSAYS OF VIRUSES

In dealing with virtually all aspects of viruses and viral diseases, there comes a time when it is necessary to determine 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. With the greater use of antivirals in treating viral infections, it is now commonplace to assess effectiveness by determining the viral load in clinical specimens. 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 basically two types of viral quantification tests—biological and physical. Tests performed on the same sample with different techniques will in some cases give vastly 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 plaque-forming unit (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 2.3). The reasons for the higher number of physical particles as compared with infectious particles are virus and assay dependent: (1) the assembly process for complete virus particles is inefficient, and morphologically complete particles are formed without the correct nucleic acid component; (2) the replication process is highly error prone (RNA viruses), and virus stocks contain particles with lethal mutations in the incorporated nucleic acid; (3) virus stocks are produced or maintained under suboptimum conditions such that infectious particles are inactivated; (4) tests for infectivity are performed in animals or cells that are not optimum for detecting infectious particles; (5) 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 (Table 2.3).

**Table 2.3 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$_{50}$ |
| Quantal infectivity assay by plaque formation | $10^8$ pfu |
| Hemagglutination assay               | $10^3$ HA units     |

**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 routinely carried out, because of the need for expensive equipment and highly trained technicians. For accuracy, the number of virus particles seen by electron microscopy must be compared with a known concentration of a standard particle such as latex beads that are added to the sample. This controls the sample volume variations that occur when preparing the samples on the solid matrix used for the procedure. Knowing the dilution of the virus preparation and the sample volume, one can calculate the concentration of virus particles. This procedure is most accurate for those viruses 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—empty capsids as opposed to complete particles.

**Hemagglutination**

As mentioned previously, some viruses can bind to red blood cells and produce an agglutination reaction: binding of virus to the red blood cells produces a lattice of cross-linked red blood cells. For this physical reactions to be visually detectable, the concentration of influenza virus particles must be in the range of $10^8$/ml for a 0.5% chicken red blood cell suspension. A relative concentration of virus can be determined by serially diluting the sample and mixing the dilutions with red blood cells. The inverse of the greatest dilution that completely agglutinates the red blood cells is defined as the “HA titer” of the virus suspension (Figure 2.13). For influenza A viruses, this is a rapid way to assess the growth of the virus, keeping in mind the lower limits of detection. Also, this procedure does not require an intact or infectious virus particle to effect the agglutination reaction. Lipid micelles with the HA protein inserted are equally effective in agglutinating red blood cells.
Quantitative Polymerase Chain Reaction Assays

With the development of real-time PCR assays (see Chapter 5), it is now possible to determine the concentration of target nucleic acid in a test sample with proper controls and with special sample preparation. 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 non-virion 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 non-virion nucleic acid—virion nucleic acid being protected by the intact virus particle. With copy number controls in the assay system, the concentration of 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 does not relate to 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 test 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. For the bacteriophage assay, serial 10-fold dilutions of a virus sample are made in a bacterial culture medium. To these diluted samples are added a host bacterial suspension in a semi-solid culture medium (melted agar). This mixture is quickly poured onto a nutrient agar bacterial culture plate to distribute the bacterial suspension evenly. The agar hardens, preventing the movement of the initially seeded bacteria. With incubation, the host bacteria divide and produce a visible “lawn” of bacteria over the surface of the agar plate. In the control plates, there are no clear areas (plaques) in the bacterial lawn. If a high percentage of the bacterial cells are infected, the entire plate can be cleared, as all bacteria are killed by the phage. Serial dilution of the phage preparation facilitates the counting of discrete plaques so that, knowing the dilution, volume tested, and the plaque count, the concentration (titer) in the original sample can be determined. In 1953, the phage test was modified for use with the newly developed tissue culture system and animal viruses. Those viruses that produce a cytopathic effect in infected cells produced discrete “holes” in the monolayers that could be readily visualized when stained with vital dyes (Figure 2.14). More recently, immunohistochemical staining procedures have been used to develop plaque assays with non-cytopathic viruses.

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 factors—that is, the plaque number followed a one-hit kinetic curve. This is not the case for viral load quantification by real-time PCR.
for many plant viruses, in which segmented genomes are incorporated into separate virus particles. Plaque assays were also instrumental in early studies of viral genetics, as plaque variants either occurring naturally or induced chemically could be selected (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 those viruses that do not produce plaques, the quantification of virus stocks was achieved by inoculating either test animals or embryonated eggs. As with plaque assays, serial dilutions of the sample or specimen were used to infect test animals or eggs. A successful infection could be scored directly—death of the animal or egg—or indirectly by showing 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 show infection. At some intermediate dilution only some of the animals or eggs would show infection. Two methods were devised (Reed–Muench or Spearman–Karber) to calculate the dilution of the virus that would infect 50% of the test animals, and the titer of the stock virus was expressed as an infectious dose 50 (ID₅₀). Although not as accurate as plaque assays and not as amenable to statistical analysis, the TCID₅₀ endpoint system is easier to set up and automate than the plaque assay.

**SPECIAL CASE OF DEFECTIVE INTERFERING MUTANTS**

As noted previously, not all physical virus particles can initiate a productive infection. A special class of defective particles—defective interfering particles—has been demonstrated *in vitro* in most families of viruses. These mutants cannot replicate by themselves, but need the presence of the parental wild-type virus; at the same time they can interfere with and usually decrease the yield of the parental virus. All defective interfering particles of RNA viruses that have been characterized are deletion mutants. In influenza viruses and reoviruses, which have segmented genomes, defective virions lack one or more of the larger segments and contain instead smaller segments consisting of an incomplete portion of the encoded gene(s). In the case of viruses with a non-segmented genome, defective interfering particles contain RNA that is shortened: as much as two-thirds of the genome may have been deleted in the defective interfering particles of vesicular stomatitis viruses. Morphologically, defective interfering particles usually

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**TABLE 2.4 Data for Calculating TCID₅₀ Endpoints**

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

For TCID₅₀ 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:

\[
\text{% mortality at dilution next above } 50\% = \frac{\text{% mortality at dilution next above } 50\%}{\text{% mortality at dilution next below}}
\]

This gives the proportional distance between the dilutions spanning the 50% endpoint. For the data in Table 2.4, this gives:

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

Adding this proportional factor to the dilution next above 50% (10⁻⁵) yields a dilution of 10⁻⁵.₅ to give one TCID₅₀/50 μl (test volume). The reciprocal of this value, adjusting for the sample volume, gives the titer of the virus stock as: 5 × 10⁶ TCID₅₀/ml.
resemble the parental virions; however, with vesicular stomatitis viruses, their normally bullet-shaped virions are shorter than wild-type virions. In the jargon used to describe these particles, normal vesicular stomatitis virions are called B particles and the defective interfering particles are called truncated or T particles.

In cell culture, the concentration of defective interfering particles increases greatly with serial passage at a high multiplicity of infection—that is, infection of a cell with a high number of virus particles. This increase in defective interfering particles is the result of several possible mechanisms: (1) their shortened genomes require less time to be replicated; (2) they are less often diverted to serve as templates for transcription of mRNA; (3) they have enhanced affinity for the viral replicase, giving them a competitive advantage over their full-length counterparts. These features also explain why defective interfering particles interfere with the replication of infectious virions with full-length RNA genomes, with progressively greater efficiency on serial passage. Production of defective interfering particles can be cell-line-dependent, in that some cell types produce more of these unique particles than others with the same virus growth conditions. It is possible that the generation of these particles is part of the host-cell defense system resulting in the production of fewer infectious particles.

The generation of other defective DNA virus genomes can occur by any of a great variety of modes of DNA rearrangement, thus defective interfering particles may contain reiterated copies of the genomic origins of replication, sometimes interspersed with DNA of host-cell origin.

Our knowledge of defective interfering particles derives mostly from studies of viral infections of cultured cells, and evidence for their role in the pathogenesis of in-vivo infections is very limited. In experimental animal studies, inoculation with defective interfering particles and infectious virus can show some decrease in virulence, but whether this can occur naturally is unknown. One well defined instance of a defective particle being linked to disease expression occurs in cattle persistently infected with bovine viral diarrhea virus, in which the defective particle generates expression of the NS3 protein that is linked to the development of mucosal disease. In this unique situation where the animal is persistently infected with a wild-type virus, many cells can harbor both the defective genome and the complete viral genome. The non-defective virus facilitates expression of the NS3 protein sequence encoded by the defective genome. Expression of the viral NS3 protein induces cell death (cytopathology) in-vitro, and expression of NS3 is characteristic of all viruses that produce the mucosal disease syndrome in cattle. Defective interfering mutants may be involved in a variety of chronic animal diseases, but because their defective and variable nature makes them difficult to detect in animals, their role in disease is still obscure.