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CHAPTER 4

Replication

WHAT'S IN THIS CHAPTER?

- We start with an overview of virus replication, then move on to look at how studying bacteriophages has helped us understand more complex viruses that are harder to work with.
- Then we go through the processes involved in virus replication step by step.
- Along the way, we start to consider whether knowledge of these processes can be used to combat virus infections.

CONTENTS

OVERVIEW OF VIRUS REPLICATION

Understanding the details of virus replication is very important. This is not just for academic reasons, but also because this knowledge provides the key to fighting virus infections. We now think about viruses in different way from our ancestors, and the way viruses are classified has been altered as our perception of them has changed:

- **By disease:** Many early civilizations, such as those of ancient Egypt and Greece, were well aware of the pathogenic effects of many different viruses. From these ancient times we have several surprisingly accurate descriptions of diseases of humans, animals, and crops, although the nature of the agents responsible for these calamities was not realized at the time. Accurate though these descriptions are, a major problem with classification according to disease is that many diverse viruses cause similar symptoms; for example, respiratory infections with fever may be caused by many different viruses.
- **By morphology:** As increasing numbers of viruses were isolated and analysis techniques improved, it became possible from the 1930s to classify viruses based on the structure of virus particles. Although this is an improvement on the previous scheme, there are still problems in distinguishing among

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viruses that are morphologically similar but cause disparate clinical symptoms (e.g., the various picornaviruses). During this era, serology became an important aid in virus classification, and particle morphology continues to be an important aspect of virus classification.

- **Functional classification:** In recent years, more emphasis has been placed on the replication strategy of the virus. This is particularly true for the composition and structure of the virus **genome** and the constraints that they impose on replication. Molecular analysis of virus genomes permits rapid and unequivocal identification of individual virus strains but can also predict the properties of a previously unknown virus with a familiar genome structure. In a teleological sense (i.e., crediting an inanimate organism such as a virus with a conscious purpose), the sole objective of a virus is to replicate its genetic information. The nature of the virus genome is therefore paramount in determining what steps are necessary to achieve this. In reality, a surprising amount of variation can occur in these processes, even for viruses with similar genome structures. The reason for this lies in compartmentalization, both of **eukaryotic** cells into nuclear and cytoplasmic compartments, and of genetic information and biochemical capacity between the virus genome and that of the host cell.

The type of cell infected by the virus has a profound effect on the process of replication. For viruses of **prokaryotes**, replication to some extent reflects the relative simplicity of their host cells. For viruses with eukaryotic hosts, processes are frequently more complex. There are many examples of animal viruses undergoing different replicative cycles in different cell types; however, the coding capacity of the virus **genome** forces all viruses to choose a strategy for replication. This might be one involving heavy reliance on the host cell, in which case the virus genome can be very compact and need only encode the essential information for a few proteins (e.g., parvoviruses). Alternatively, large and complex virus genomes, such as those of poxviruses, encode most of the information necessary for replication, and the virus is only reliant on the cell for the provision of energy and the apparatus for macromolecular synthesis, such as ribosomes (see Chapter 1).

Viruses with an RNA lifestyle (i.e., an RNA genome plus messenger RNAs) have no apparent need to enter the nucleus, although during the course of replication a few do. DNA viruses, as might be expected, mostly replicate in the nucleus, where host-cell DNA is replicated and where the biochemical apparatus necessary for this process is located. However, some viruses with DNA genomes (e.g., poxviruses) have evolved to contain sufficient biochemical capacity to be able to replicate in the cytoplasm, with minimal requirement for host-cell functions. Most of this chapter will examine the process of virus replication and will look at some of the variations on the basic theme.

INVESTIGATION OF VIRUS REPLICATION

Bacteriophages have long been used by virologists as models to understand the biology of viruses. This is particularly true of virus replication. Two very significant experiments that illustrated the fundamental nature of viruses were performed on bacteriophages. The first of these was done by Ellis and Delbruck in 1939 and is usually referred to as the single-burst experiment or one-step growth curve (Figure 4.1). This was the first experiment to show the three essential phases of virus replication:

- Initiation of infection
- Replication and expression of the virus genome
- Release of mature **virions** from the infected cell

In this experiment, bacteriophage particles were added to a culture of rapidly growing bacteria, and after a period of a few minutes, the culture was diluted, effectively preventing further interaction between the **phage** particles and the cells. This simple step is the key to the entire experiment, because it effectively synchronizes the infection of the cells and allows the subsequent phases of replication in a population of individual cells and virus particles to

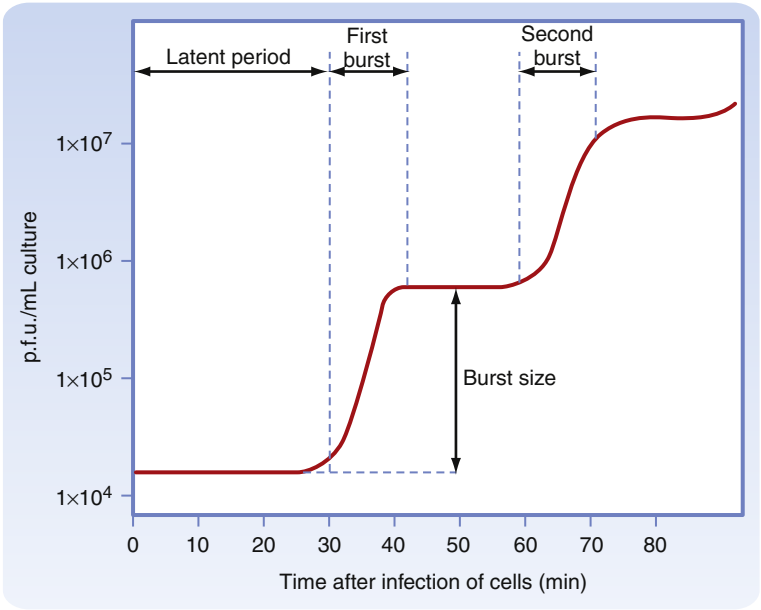

FIGURE 4.1 **The one-step growth curve or single-burst experiment.**
First performed by Ellis and Delbruck in 1939, this classic experiment illustrates the true nature of virus replication. Details of the experiment are given in the text. Two bursts (crops of phage particles released from cells) are shown in this particular experiment.

be viewed as if they were a single interaction (in much the same way that molecular cloning of nucleic acids allows analysis of populations of nucleic acid molecules as single species). Repeated samples of the culture were taken at short intervals and analyzed for bacterial cells by plating onto agar plates and for phage particles by plating onto lawns of bacteria. As can be seen in Figure 4.1, there is a stepwise increase in the concentration of phage particles with time, each increase in phage concentration representing one replicative cycle of the virus. However, the data from this experiment can also be analyzed in a different way, by plotting the number of **plaque-forming units** (**p.f.u.**) per bacterial cell against time (Figure 4.2). In this type of assay, a plaque-forming unit can be either a single extracellular virus particle or an infected bacterial cell. These two can be distinguished by disruption of the bacteria with chloroform before plating, which releases any intracellular phage particles, thus providing the total virus count (i.e., intracellular plus extracellular particles).

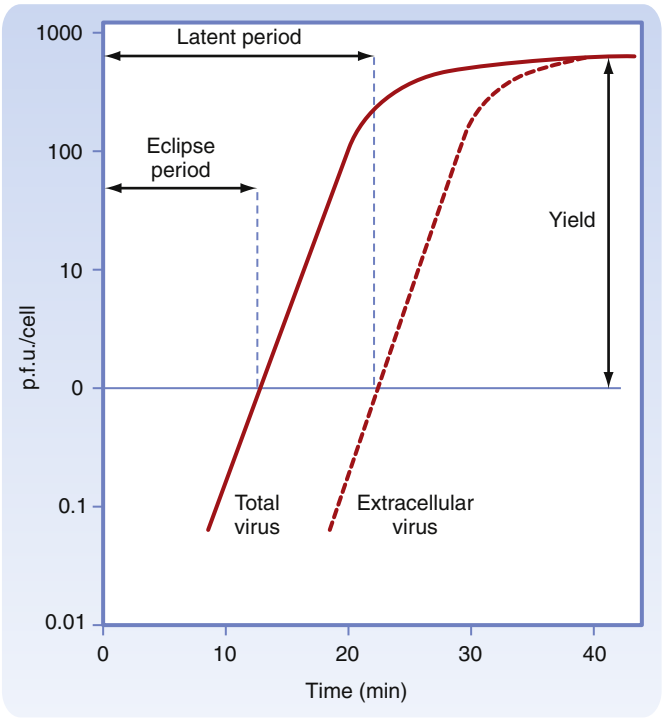

FIGURE 4.2 **Analysis of data from a single-burst experiment.**
Unlike Figure 4.1, which shows the total number of plaque-forming units (p.f.u.) produced, here the data are plotted as p.f.u./bacterial cell, reflecting the events occurring in a typical infected cell in the population. The phases of replication named on the graph are defined in the text.

Several additional features of virus replication are visible from the graph in Figure 4.2. Immediately after dilution of the culture, there is a phase of 10 to 15 minutes when no phage particles are detectable; this is known as the **eclipse period**. This represents a time when virus particles have broken down after penetrating cells, releasing their **genomes** as a prerequisite to replication. At this stage, they are no longer infectious and therefore cannot be detected by the plaque assay. The **latent period** is the time before the first new extracellular virus particles appear and is around 20 to 25 minutes for many bacteriophages. About 40 minutes after the cells have been infected, the curves for the total number of virus particles and for the extracellular virus merge because the infected cells have lysed and released any intracellular phage particles by this time. The yield (i.e., number) of particles produced per infected cell can be calculated from the overall rise in phage **titre**.

Following the development of **plaque** assays for animal viruses in the 1950s, single-burst experiments have been performed for many viruses of **eukaryotes** with similar results (Figure 4.3). The major difference between these viruses and bacteriophages is the much longer time interval required for replication, which is measured in terms of hours and, in some cases, days, rather than minutes. This difference reflects the much slower growth rate of eukaryotic cells and, in part, the complexity of virus replication in compartmentalized cells. Biochemical analysis of virus replication in eukaryotic cells has also been used to analyze the levels of virus and cellular protein and nucleic acid synthesis and to examine the intracellular events occurring during synchronized infections (Figure 4.4). The use of various metabolic inhibitors also proved to be a valuable tool in such experiments. Examples of the use of such drugs will be discussed later in this chapter.

The second key experiment on virus replication using **bacteriophages** was performed by Hershey and Chase in 1952. Bacteriophage T2 was propagated in *Escherichia coli* cells that had been labeled with one of two radioisotopes, either ^{35}S, which is incorporated into sulphur-containing amino acids in proteins, or ^{32}P, which is incorporated into nucleic acids (which do not contain any sulphur; Figure 4.5). Particles labeled in each of these ways were used to infect bacteria. After a short period to allow attachment to the cells, the mixture was homogenized briefly in a blender, which did not destroy the bacterial cells but was sufficiently vigorous to knock the phage coats off the outsides of the cells. Analysis of the radioactive content in the cell pellets and culture supernatant (containing the empty phage coats) showed that most of the radioactivity in the ^{35}S-labeled particles remained in the supernatant, while in the ^{32}P-labeled particles most of the radiolabel had entered the cells. This experiment proves that it was the DNA **genome** of the bacteriophage that entered the cells and initiated the infection rather than any other component (such as proteins). Although it might seem obvious now, at the time this experiment settled a great

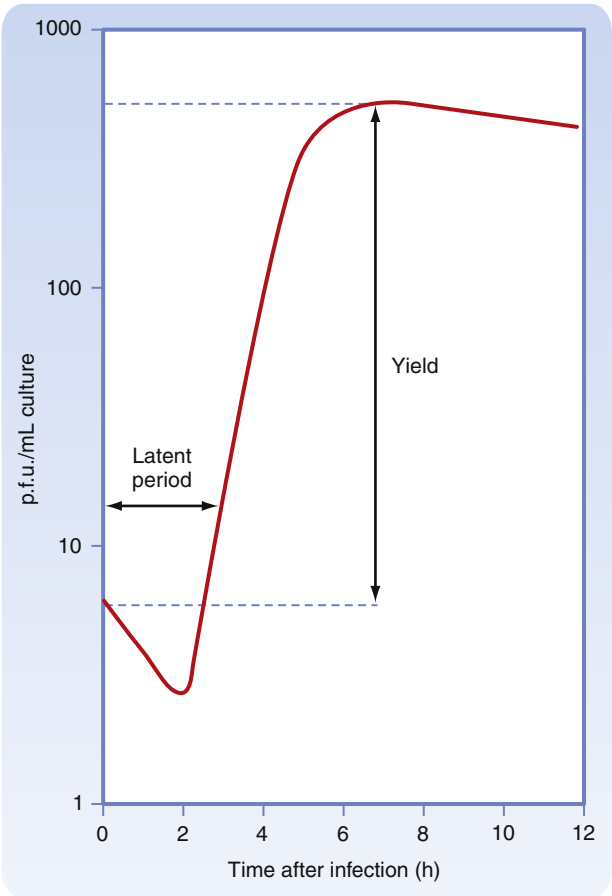

FIGURE 4.3 **Replication of lytic eukaryotic viruses occurs in a similar way to that of bacteriophages.**
This figure shows a single-burst type of experiment for a picornavirus (e.g., poliovirus). This type of data can only be produced from synchronous infections where a high **multiplicity of infection** is used.

controversy over whether a structurally simple polymer such as a nucleic acid, which was known to contain only four monomers, was complex enough to carry genetic information. (At the time, it was generally believed that proteins, which consist of a much more complex mixture of more than 20 different amino acids, were the carriers of the genes and that DNA was probably a structural component of cells and viruses.) Together, these two experiments illustrate the essential processes of virus replication. Virus particles enter susceptible cells and release their genomic nucleic acids. These are replicated and packaged into virus particles consisting of newly synthesized virus proteins, which are then released from the cell.

BOX 4.1. SEEING THE FOREST FOR THE TREES

Henry Ford said "History is bunk"—but he was wrong. Obscure experiments from the 1930s might not seem very interesting, but if you think that, you're making the same mistake as Henry. In theory, it would be very simple to repeat Ellis and Delbruck's experiment in a modern virus research laboratory, except that it's unlikely that this would happen. Bacteriophages don't make people sick (very often—more about that later in the book), so they don't get much attention these days when the only way you can run a laboratory is to get lots of research grants for working on "important" viruses such as HIV. However, if you tried to do the Ellis and Delbruck experiment on HIV, you wouldn't be able to, because of the biology of this virus. Although it goes through all the same stages of replication as a bacteriophage, you wouldn't be able to interpret the data you got because of the kinetics. Bacteriophages have easily been the most important model organisms in virology, and continue to give us insights into diversity, adaptation, and virulence, which are much harder to study in more advanced viruses.

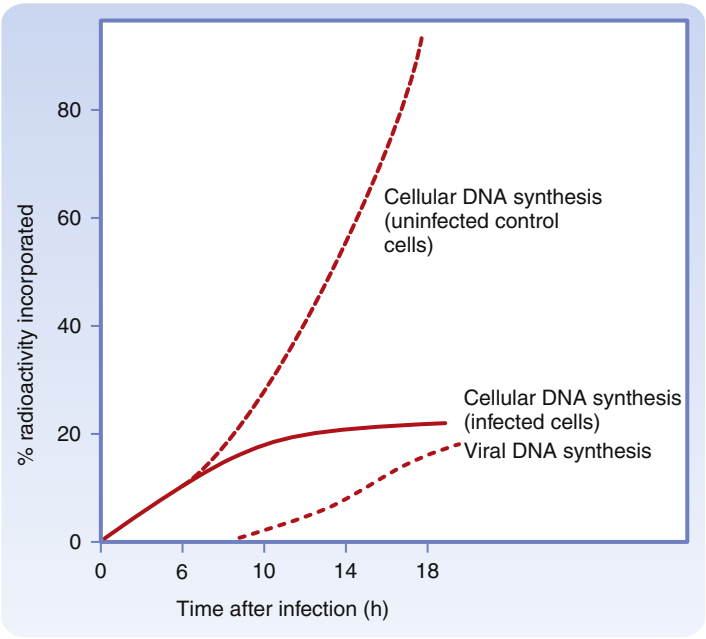

FIGURE 4.4 **Biochemistry of virus infection.**
This graph shows the rate of cellular and virus DNA synthesis (based on the incorporation of radiolabelled nucleotides into high-molecular-weight material) in uninfected and virus-infected cells.

THE REPLICATION CYCLE

Virus replication can be divided into eight stages, as shown in Figure 4.6. These are purely arbitrary steps, used here for convenience in explaining the replication cycle of a nonexistent typical virus. For simplicity, this chapter concentrates on viruses

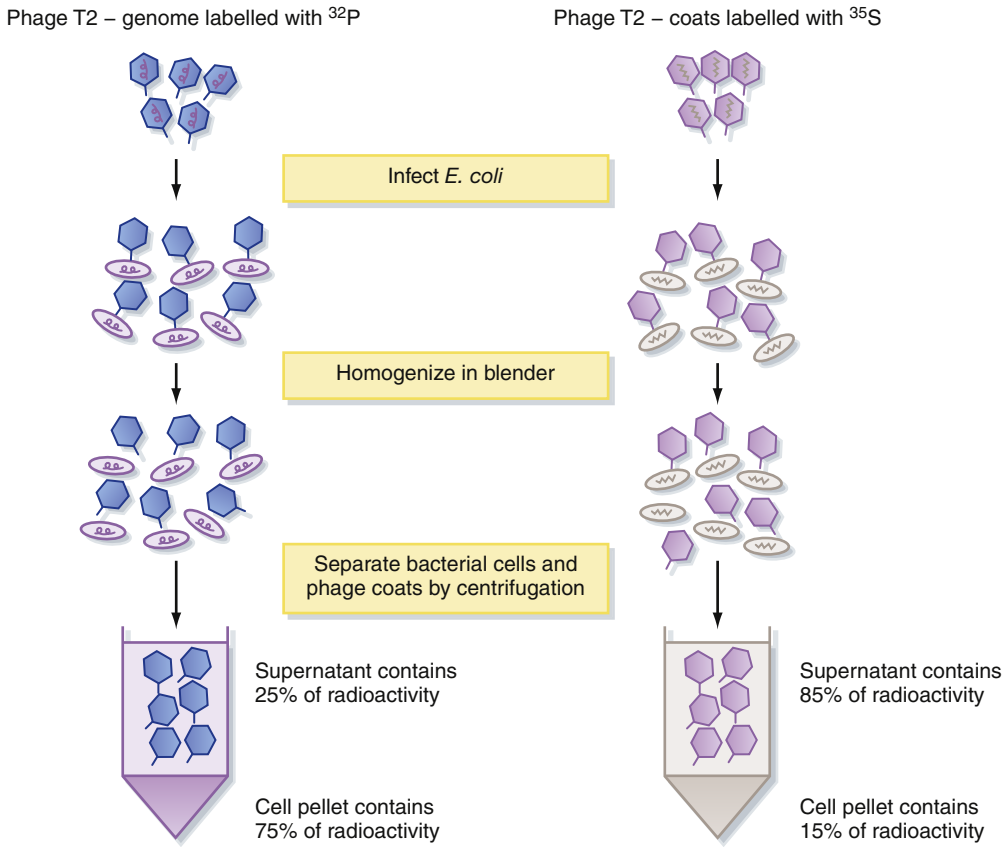

FIGURE 4.5 **The Hershey—Chase experiment.**
The Hershey—Chase experiment, first performed in 1952, demonstrated that virus genetic information was encoded by nucleic acids and not proteins. Details of the experiment are described in the text.

that infect vertebrates. Viruses of bacteria, invertebrates, and plants are mentioned briefly, but the overall objective of this chapter is to illustrate similarities in the pattern of replication of different viruses. Regardless of their hosts, all viruses must undergo each of these stages in some form to successfully complete their replication cycles. Not all the steps described here are detectable as distinct stages for all viruses; often they blur together and appear to occur almost simultaneously. Some of the individual stages have been studied in great detail, and a tremendous amount of information is known about them. Other stages have been much more difficult to study, and considerably less information is available.

Attachment

Because the separate stages of virus replication described here are arbitrary and because complete replication necessarily involves a cycle, it is possible to begin

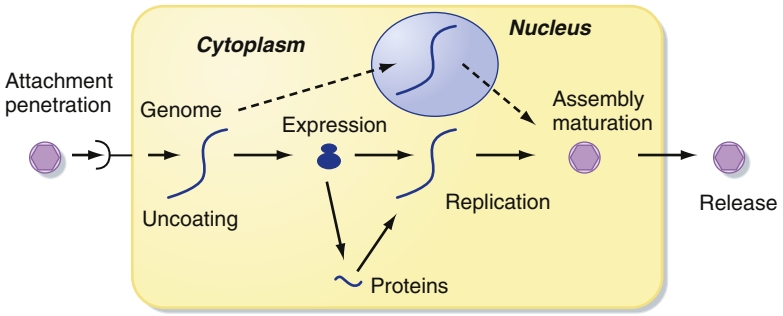

FIGURE 4.6 **A generalized scheme for virus replication.**
This diagram shows an outline of the steps that occur during replication of a "typical" virus that infects eukaryotic cells. See the text for more details.

discussion of virus replication at any point. Arguably, it is most logical to consider the first interaction of a virus with a new host cell as the starting point of the cycle. Technically, virus **attachment** consists of specific binding of a **virus-attachment protein** (or antireceptor) to a cellular **receptor** molecule. Many examples of virus receptors are now known (see Figure 4.7 and "Further Reading" at the end of this chapter). The target receptor molecules on cell surfaces may be proteins (usually glycoproteins) or the carbohydrate structures present on glycoproteins or glycolipids. The former are usually specific receptors in that a virus may use a particular protein as a receptor. Carbohydrate groups are usually less specific because the same configuration of sugar side-chains may occur on many different glycosylated membrane-bound molecules. Some complex viruses (e.g., poxviruses, herpesviruses) use more than one

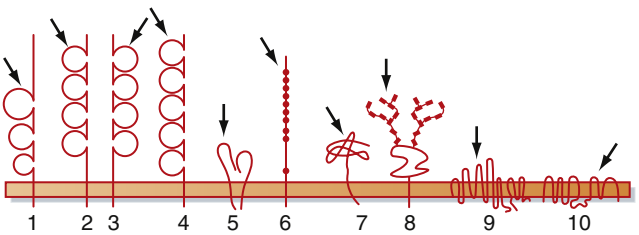

FIGURE 4.7 **Virus receptors.**
The arrows in this figure indicate approximate virus attachment sites. (1) Poliovirus receptor (PVR). (2) CD4: HIV. (3) Carcinoembryonic antigen(s): MHV (coronavirus). (4) ICAM-1: most rhinoviruses. (Note that 1 to 4 are all immunoglobulin superfamily molecules.) (5) VLA-2 integrin: ECHO viruses. (6) LDL receptor: some rhinoviruses. (7) Aminopeptidase N: coronaviruses. (8) Sialic acid (on glycoprotein): influenza, reoviruses, rotaviruses. (9) Cationic amino acid transporter: murine leukemia virus. (10) Sodium-dependent phosphate transporter: Gibbon ape leukemia virus.

receptor and therefore have alternative routes of uptake into cells. Virus receptors fall into many different classes (e.g., immunoglobulin-like superfamily molecules, membrane-associated receptors, and transmembrane transporters and channels). The one factor that unifies all virus receptors is that they did not evolve and are not manufactured by cells to allow viruses to enter cells; rather, viruses have subverted molecules required for normal cellular functions.

Plant viruses face special problems initiating infection. The outer surfaces of plants are composed of protective layers of waxes and pectin, but more significantly, each cell is surrounded by a thick wall of cellulose overlying the cytoplasmic membrane. To date, no plant virus is known to use a specific cellular receptor of the type that animal and bacterial viruses use to attach to cells. Instead, plant viruses rely on a breach of the integrity of a cell wall to introduce a virus particle directly into a host cell. This is achieved either by the vector associated with transmission of the virus or simply by mechanical damage to cells. After replication in an initial cell, the lack of receptors poses further problems for plant viruses in recruiting new cells to the infection. This is discussed in Chapter 6.

Some of the best understood examples of virus–receptor interactions are from the *Picornaviridae*. The virus–receptor interaction in Picornaviruses has been studied intensively from the viewpoint of both the structural features of the virus responsible for receptor binding and those of the receptor molecule. The major human rhinovirus (HRV) receptor molecule, ICAM-1 (intercellular adhesion molecule 1 or CD54) is an adhesion molecule whose normal function is to bind cells to adjacent substrates. Structurally, ICAM-1 is similar to an immunoglobulin molecule, with constant (C) and variable (V) domains homologous to those of antibodies, and is regarded as a member of the immunoglobulin superfamily of proteins (Figure 4.7). Similarly, the poliovirus receptor (PVR or CD155) is an integral membrane protein that is also a member of this family, with one variable and two constant domains, which is involved in establishment of intercellular junctions between epithelial cells.

Since the structure of a number of picornavirus **capsids** is known at a resolution of a few angstroms (Chapter 2), it has been possible to determine the features of the virus responsible for **receptor** binding. In human rhinoviruses (HRVs), there is a deep cleft known as the canyon in the surface of each triangular face of the **icosahedral** capsid, which is formed by the flanking monomers, VP1, VP2, and VP3 (Figure 4.8). Biochemical evidence from a class of inhibitory drugs that block attachment of HRV particles to cells indicates that the interaction between ICAM-1 and the virus particle occurs on the floor of this canyon. Unlike other areas of the virus surface, the amino acid residues forming the

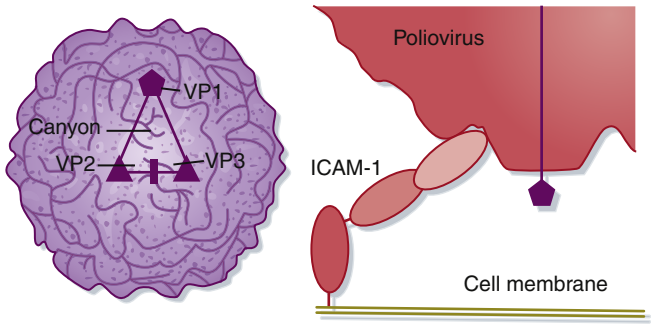

FIGURE 4.8 **Rhinovirus receptor binding.**
Rhinovirus particles have a deep surface cleft, known as the canyon, between the three monomers (VP1, 2, and 3) making up each face of the particle.

internal surfaces of the canyon are relatively invariant. It was suggested that these regions are protected from antigenic pressure because the antibody molecules are too large to fit into the cleft. This is important because radical changes here, although allowing the virus to escape an immune response, would disrupt receptor binding. Subsequently, it has been found that the binding site of the receptor extends well over the edges of the canyon, and the binding sites of neutralizing antibodies extend over the rims of the canyon. Nevertheless, the residues most significant for the binding site of the receptor and for neutralizing antibodies are separated from each other. In polioviruses, there is a similar canyon that runs around each five-fold vertex of the capsid. The highly variant regions of the capsid to which antibodies bind are located on the peaks on either side of this trough, which is again too narrow to allow antibody binding to the residues at its base. The invariant residues at the sides of the trough interact with the receptor.

Even within the *Picornaviridae* there is considerable variation in receptor usage. Although 90 serotypes of HRV use ICAM-1 as their receptor, some 10 serotypes use proteins related to the low-density lipoprotein (LDL) receptor. Encephalomyocarditis virus (EMCV) has been reported to use the immunoglobulin molecule vascular cell adhesion factor (VCAM-1) or glycophorin A. Several picornaviruses use other integrins as receptors: some enteric cytopathic human orphan (ECHO) viruses use VLA-2 or fibronectin, and foot-and-mouth disease viruses (FMDVs) have been reported to use an unidentified integrin-like molecule. Other ECHO viruses use complement decay-accelerating factor (DAF, CD55), a molecule involved in complement regulation. This list is given to illustrate that even within one structurally closely related family of viruses, there is considerable variation in the receptor structures used.

Another well-studied example of a virus–receptor interaction is that of influenza virus. The influenza virus hemagglutinin protein forms one of the two types of glycoprotein spikes on the surface of the particles (see Chapter 2), the other type being formed by the neuraminidase protein. Each hemagglutinin spike is composed of a trimer of three molecules, while the neuraminidase spike consists of a tetramer (Figure 4.9). The hemagglutinin spikes are responsible for binding the influenza virus receptor, which is sialic acid (*N*-acetyl neuraminic acid), a sugar group commonly found on a variety of glycosylated molecules. As a result, little cell-type specificity is imposed by this receptor interaction so influenza viruses bind to a wide variety of different cell types (e.g., causing **hemagglutination** of red blood cells) in addition to the cells in which **productive infection** occurs.

The neuraminidase molecule of influenza virus and paramyxoviruses illustrates another feature of this stage of virus replication. Attachment to cellular receptors is in most cases a reversible process—if **penetration** of the cells does not ensue, the virus can elute from the cell surface. Some viruses have specific mechanisms for detachment, and the neuraminidase protein is one of these. Neuraminidase is an esterase that cleaves sialic acid from sugar side-chains. This is particularly important for influenza. Because the receptor molecule is so widely distributed, the virus tends to bind inappropriately to a variety of cells

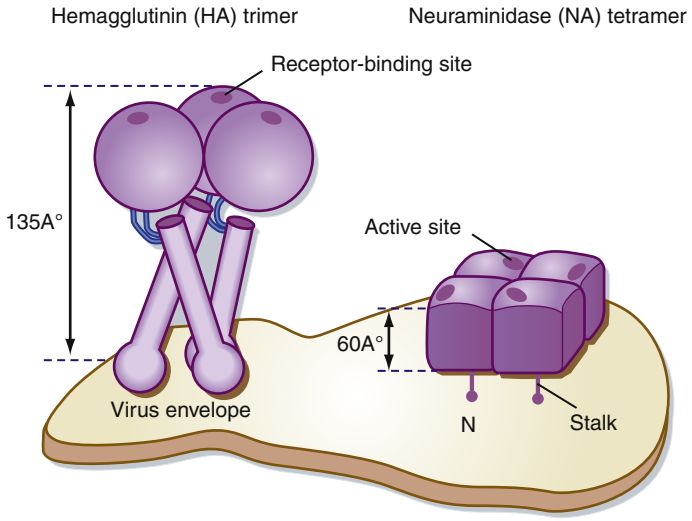

FIGURE 4.9 **Influenza virus glycoprotein spikes.**
The glycoprotein spikes on the surface of influenza virus (and many other enveloped viruses) are multimers consisting of three copies of the hemagglutinin protein (trimer) and four copies of the neuraminidase protein (tetramer).

and even cell debris; however, elution from the cell surface after receptor binding has occurred often leads to changes in the virus (e.g., loss or structural alteration of **virus-attachment protein**) that decrease or eliminate the possibility of subsequent attachment to other cells. Thus, in the case of influenza, cleavage of sialic acid residues by neuraminidase leaves these groups bound to the active site of the hemagglutinin, preventing that particular molecule from binding to another receptor.

BOX 4.2. WHY DO THESE OBSCURE DETAILS MATTER?

I've spent quite a long time in this chapter describing the interactions of certain viruses with their receptors. If you look at the research literature about virus receptors, you'll find it's huge. Why all the fuss? It's because this first interaction of a virus particle with a host cell is in some ways the most important step in replication—it goes a long way to determining what happens in the rest of the process. For one thing, if a cell has no receptors for a virus, it doesn't get infected (and vice versa). So tropism, the ability to infect a particular cell type, is largely controlled by receptor interactions. Going on from there, small changes can have big effects, so this process is important to understand in detail. At present, the H5N1 type of influenza virus can infect humans and when it does, it's likely to kill them, but it really struggles to do this because at the moment, it's really a bird (avian) virus. With a very small change in the receptor usage, H5N1 could become a deadly human virus. In addition, when you understand these processes, you can use them against the virus. We've had anti-influenza drugs for decades, but they weren't very good. Modern influenza drugs such as Tamiflu and Relenza inhibit the neuraminidase protein involved in receptor interactions (although in release from the cell rather than uptake). If H5N1 ever does make the jump to being a human virus, we're going to need these drugs to stay alive.

In most cases, the expression (or absence) of receptors on the surface of cells largely determines the **tropism** of a virus (i.e., the type of host cell in which it is able to replicate). In some cases, intracellular blocks at later stages of replication are responsible for determining the range of cell types in which a virus can carry out a productive infection, but this is not common. Therefore, this initial stage of replication and the very first interaction between the virus and the host cell has a major influence on virus pathogenesis and in determining the course of a virus infection. In some cases, interactions with more than one protein are required for virus entry. These are not examples of alternative **receptor** use, as neither protein alone is a functional receptor—both are required to act together. An example is the process by which adenoviruses enter cells. This requires a two-stage process involving an initial interaction of the virion fiber protein with a range of cellular receptors, which include the major histocompatibility complex class I (MHC-I) molecule and

the coxsackievirus–adenovirus receptor (CAR). Another virion protein, the penton base, then binds to the integrin family of cell-surface proteins, allowing internalization of the particle by receptor-mediated endocytosis. Most cells express primary receptors for the adenovirus fiber coat protein; however, the internalization step is more selective, giving rise to a degree of cell selection.

A similar observation has been made with human immunodeficiency virus (HIV). The primary receptor for HIV is the helper T-cell differentiation antigen, CD4. Transfection of human cells that do not normally express CD4 (such as epithelial cells) with recombinant CD4-expression constructs makes them permissive for HIV infection; however, transfection of rodent cells with human CD4-expression vectors does not permit productive HIV infection—something else is missing from the mouse cells. If HIV **provirus** DNA is inserted into rodent cells by transfection, virus is produced, showing that there is no intracellular block to infection. So there must be one or more accessory factors in addition to CD4 that are required to form a functional HIV receptor. These are a family of proteins known as β-chemokine receptors. Several members of this family have been shown to play a role in the entry of HIV into cells, and their distribution may be the primary control for the **tropism** of HIV for different cell types (lymphocytes, macrophages, etc.). Furthermore, there is evidence, in at least some cell types, that HIV infection is not blocked by competing soluble CD4, indicating that in these cells a completely different receptor strategy may be being used. Several candidate molecules have been put forward to fill this role (e.g., galactosylceramide and various other candidate proteins). However, if any or all of these do allow HIV to infect a range of CD4-negative cells, this process is much less efficient than the interaction of the virus with its major receptor complex.

In some cases, specific receptor binding can be side-stepped by nonspecific or inappropriate interactions between virus particles and cells. It is possible that virus particles can be accidentally taken up by cells via processes such as pinocytosis or phagocytosis (see later). However, in the absence of some form of physical interaction that holds the virus particle in close association with the cell surface, the frequency with which these accidental events happen is very low. On occasion, antibody-coated virus particles binding to Fc receptor molecules on the surface of monocytes and other blood cells can result in virus uptake. This phenomenon has been shown to occur in a number of cases where antibody-dependent enhancement of virus uptake results in unexpected findings. For example, the presence of antivirus antibodies can occasionally result in increased virus uptake by cells and increased pathogenicity rather than virus neutralization, as would normally be expected. It has been suggested that this mechanism may also be important in the uptake of HIV by macrophages and monocytes and that this might be a factor in the

pathogenesis of acquired immune deficiency syndrome (AIDS), although this is not yet certain.

Penetration

Penetration of the target cell normally occurs a very short time after attachment of the virus to its **receptor** in the cell membrane. Unlike attachment, cell penetration is generally an energy-dependent process; that is, the cell must be metabolically active for this to occur. Three main mechanisms are involved:

- Translocation of the entire virus particle across the cytoplasmic membrane of the cell (Figure 4.10). This process is relatively rare among viruses and is poorly understood. It must be mediated by proteins in the virus **capsid** and specific membrane receptors.
- Endocytosis of the virus into intracellular vacuoles (Figure 4.11). This is probably the most common mechanism of virus entry into cells. It does not require any specific virus proteins (other than those already utilized for receptor binding) but relies on the normal formation and internalization of coated pits at the cell membrane. Receptor-mediated endocytosis is an efficient process for taking up and concentrating extracellular macromolecules.
- **Fusion** of the virus **envelope** (so this is only applicable to enveloped viruses) with the cell membrane, either directly at the cell surface or following endocytosis in a cytoplasmic vesicle (Figure 4.12), which requires the presence of a specific fusion protein in the virus envelope—for example, influenza hemagglutinin or retrovirus transmembrane (TM) glycoproteins. These proteins promote the joining of the cellular and virus membranes, which results in the **nucleocapsid** being deposited directly in the cytoplasm. There are two types of virus-driven membrane fusion: one pH dependent, the other pH independent.

The process of endocytosis is almost universal in animal cells and deserves further consideration (Figure 4.11). The formation of coated pits results in the engulfment of a membrane-bounded vesicle by the cytoplasm of the cell. The lifetime of these initial coated vesicles is very short. Within seconds, most fuse with endosomes, releasing their contents into these larger vesicles. At this point, any virus contained within these structures is still cut off from the cytoplasm by a lipid bilayer and therefore has not strictly entered the cell. Moreover, as endosomes fuse with lysosomes, the environment inside these vessels becomes increasingly hostile as the pH falls, while the concentration of degradative enzymes rises. This means that the virus particle must leave the vesicle and enter the cytoplasm before it is degraded. There are a number of mechanisms by which this can occur, including membrane **fusion** and rescue by transcytosis. The release of virus particles from endosomes and their passage into the cytoplasm is intimately connected

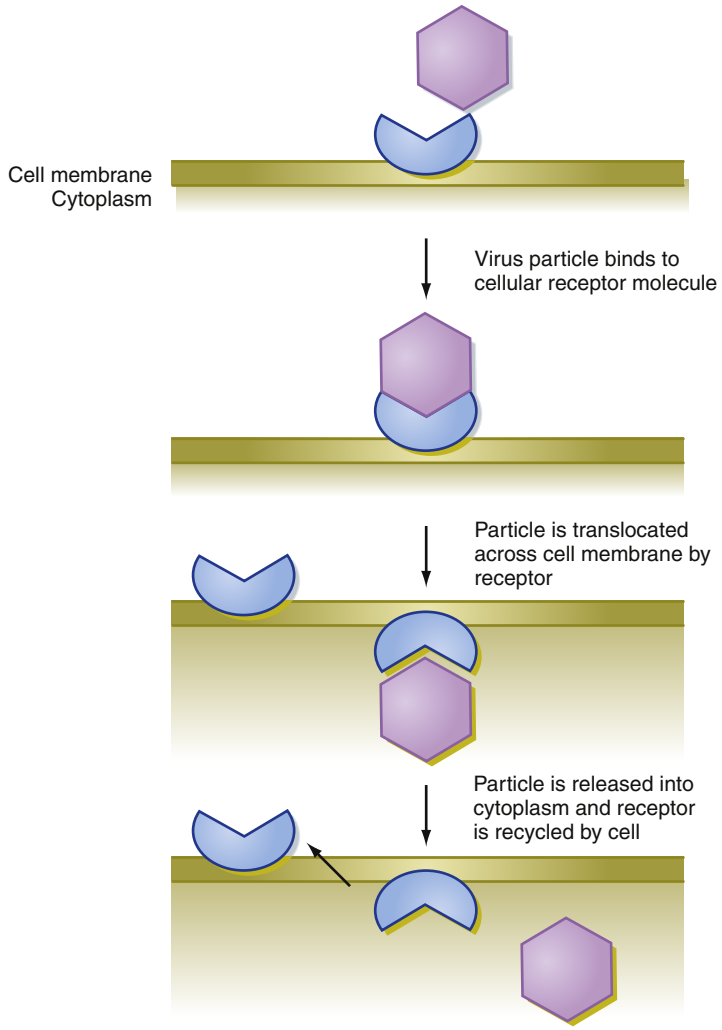

FIGURE 4.10 **Translocation of virus particles.**
Translocation of entire virus particles across the cell membrane by cell-surface receptors.

with (and often impossible to separate from) the process of uncoating (see next).

Uncoating

Uncoating is a general term for the events that occur after **penetration**, in which the virus **capsid** is completely or partially removed and the virus **genome** is exposed, usually in the form of a nucleoprotein complex. Unfortunately this is one of the stages of virus replication that has been least studied and is relatively

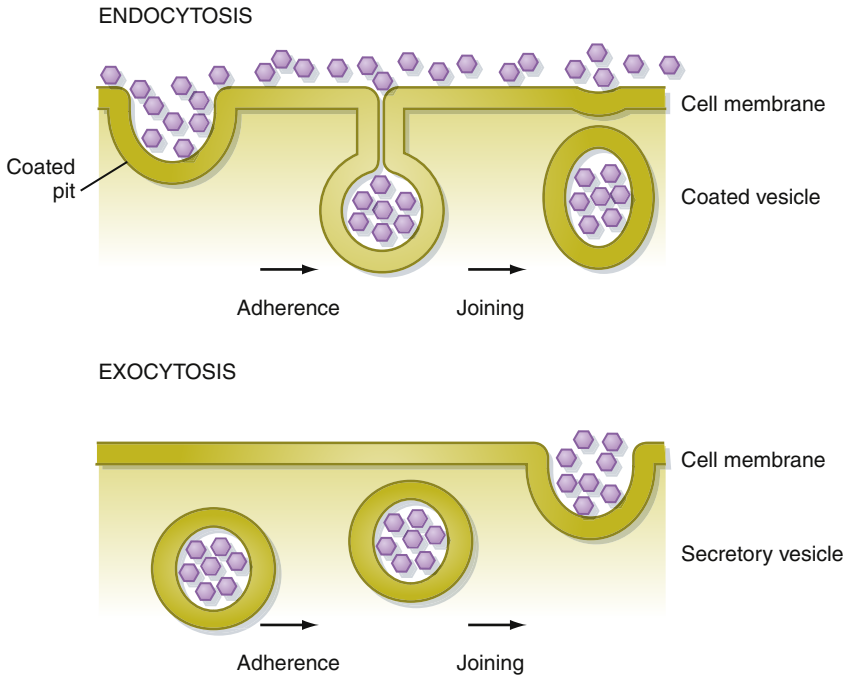

FIGURE 4.11 **Endocytosis and exocytosis of virus particles.**
The processes of endocytosis and exocytosis are involved in both the takeup and release of enveloped virus particles from host cells. Viruses modify these normal cellular processes by encoding proteins that promote endocytosis (e.g., virus attachment proteins and fusion proteins) and release from the cell surface via exocytosis (e.g., the influenza neuraminidase protein).

poorly understood. In one sense, the removal of a virus **envelope** that occurs during membrane fusion is part of the uncoating process. Fusion between virus envelopes and endosomal membranes is driven by virus fusion proteins. These are usually activated by the uncloaking of a previously hidden fusion domain as a result of conformational changes in the protein induced by the low pH inside the vesicle, although in some cases the fusion activity is triggered directly by **receptor** binding. The initial events in uncoating may occur inside endosomes, being triggered by the change in pH as the endosome is acidified, or directly in the cytoplasm. Proteins that form ion channels, or cations such as chloroquine and ammonium chloride, can be used to block the acidification of these vesicles and to determine whether events are occurring following the acidification of endosomes (e.g., pH-dependent membrane fusion) or directly at the cell surface or in the cytoplasm (e.g., pH-independent membrane fusion). Endocytosis is potentially dangerous for viruses, because if they remain in the vesicle too long they will be irreversibly damaged by acidification or lysosomal enzymes. Some viruses can control this process; for example, the

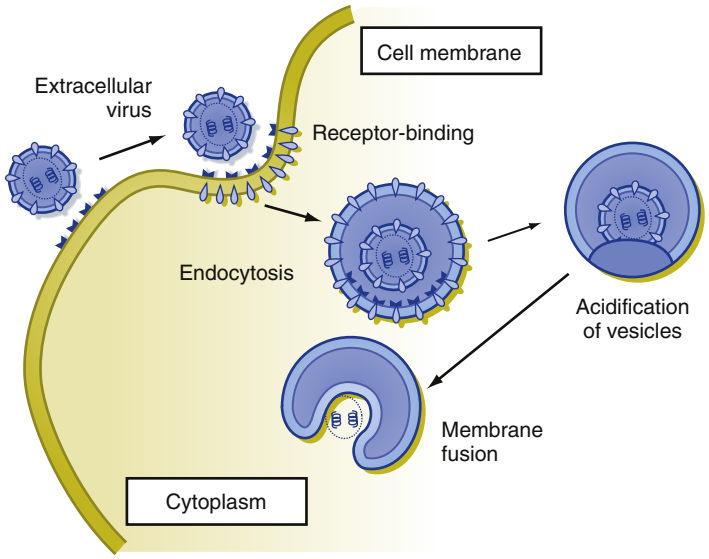

FIGURE 4.12 Virus-induced membrane fusion.
This uptake process is dependent on the presence of a specific fusion protein on the surface of the virus that, under particular circumstances (e.g., acidification of the virus-containing vesicle), becomes activated, inducing fusion of the vesicle membrane and the virus envelope.

influenza virus M2 protein is a membrane channel that allows entry of hydrogen ions into the **nucleocapsid**, facilitating uncoating. The M2 protein is multifunctional and also has a role in influenza virus **maturation** (see later).

In picornaviruses, **penetration** of the cytoplasm by exit of virus from endosomes is tightly linked to uncoating (Figure 4.13). The acidic environment of the endosome causes a conformational change in the **capsid** that reveals

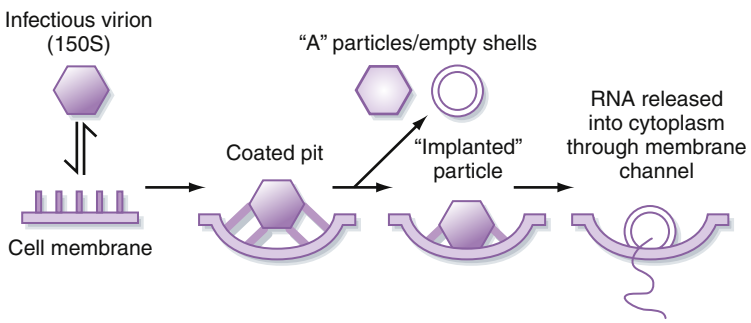

FIGURE 4.13 Cell penetration and uncoating of polioviruses.
Following receptor binding, poliovirus particles are taken up by host cells in vesicles that interact with the cytoskeleton. This is an active, energy-dependent process.

hydrophobic domains not present on the surface of mature virus particles. The interaction of these hydrophobic patches with the endosomal membrane is believed to form pores through which the **genome** passes into the cytoplasm.

The product of uncoating depends on the structure of the virus nucleocapsid. In some cases, it might be relatively simple (e.g., picornaviruses have a small basic protein of approximately 23 amino acids [VPg] covalently attached to the 5′ end of the vRNA genome), or highly complex (e.g., retrovirus cores are highly ordered nucleoprotein complexes that contain, in addition to the diploid RNA genome, the reverse transcriptase enzyme responsible for converting the virus RNA genome into the DNA **provirus**). The structure and chemistry of the nucleocapsid determines the subsequent steps in replication. As discussed in Chapter 3, reverse transcription can occur only inside an ordered retrovirus core particle and cannot proceed with the components of the reaction free in solution. Herpesvirus, adenovirus, and polyomavirus **capsids** undergo structural changes following **penetration**, but overall remain largely intact. These capsids contain sequences that are responsible for attachment to the cytoskeleton, and this interaction allows the transport of the entire capsid to the nucleus. It is at the nuclear pores that uncoating occurs and the **nucleocapsid** passes into the nucleus. In reoviruses and poxviruses, complete uncoating does not occur, and many of the reactions of genome replication are catalyzed by virus-encoded enzymes inside cytoplasmic particles that still resemble the mature **virions**.

Genome replication and gene expression

The replication strategy of any virus depends on the nature of its genetic material. In this respect, all viruses can be divided into seven groups. Such a scheme was first proposed by David Baltimore in 1971. Originally, this classification included only six groups, but it has since been extended to include the scheme of **genome** replication used by the hepadnaviruses and caulimoviruses. For viruses with RNA genomes in particular, genome replication and the expression of genetic information are inextricably linked, therefore both of these criteria are taken into account in the following scheme. The control of gene expression determines the overall course of a virus infection (acute, chronic, persistent, or latent), and such is the emphasis placed on gene expression by molecular biologists that this subject is discussed in detail in Chapter 5. A schematic overview of the major events during replication of the different virus genomes is shown in Figure 4.14, and a complete list of all the families that constitute each class is given in Appendix 2 WEB.

- **Class I:** Double-stranded DNA.
  This class can be subdivided into two further groups: (a) replication is exclusively nuclear (Figure 4.14), meaning that replication of these viruses is

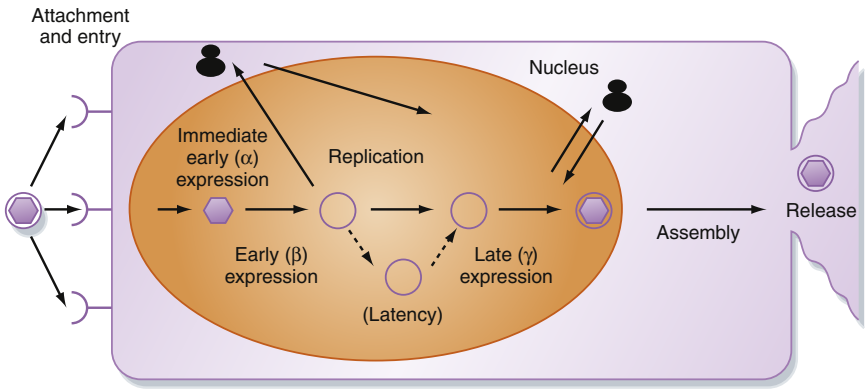

FIGURE 4.14 Schematic representation of the replication of class I viruses.
Details of the events that occur for genomes of this type are given in the text.

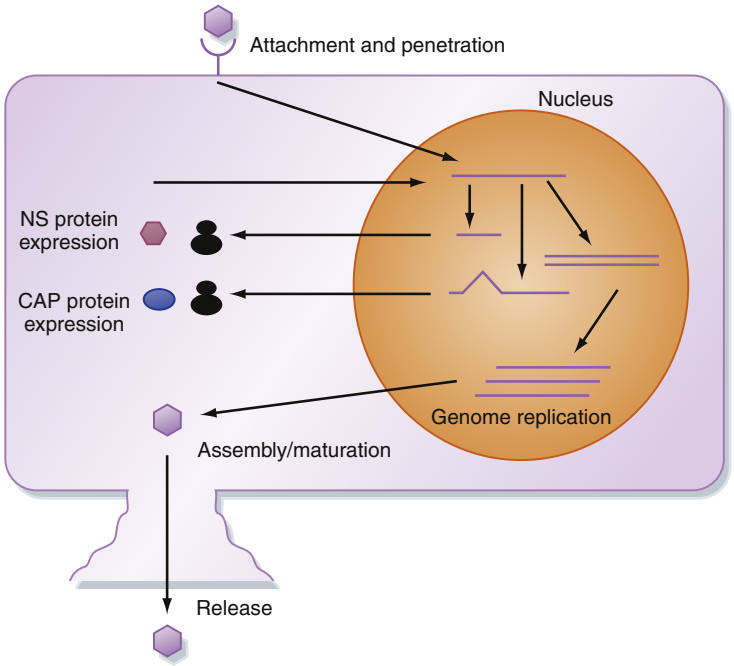

FIGURE 4.15 Schematic representation of the replication of class II viruses.
Details of the events that occur for genomes of this type are given in the text.

relatively dependent on cellular factors; (b) replication occurs in cytoplasm (e.g., the *Poxviridae*), in which case the viruses have evolved (or acquired) all the necessary factors for transcription and replication of their genomes and are therefore largely independent of the cellular machinery.

- **Class II:** Single-stranded DNA (Figure 4.15).
  Replication occurs in the nucleus, involving the formation of a double-stranded intermediate that serves as a template for the synthesis of single-stranded progeny DNA.
- **Class III:** Double-stranded RNA (Figure 4.16).
  These viruses have segmented genomes. Each segment is transcribed separately to produce individual **monocistronic** mRNAs.
- **Class IV:** Single-stranded (+)sense RNA.
  These can be subdivided into two groups: (a) viruses with **polycistronic** mRNA (Figure 4.17)—as with all the viruses in this class, the genome RNA forms the mRNA and is translated to form a **polyprotein** product, which is subsequently cleaved to form the mature proteins; (b) viruses with complex transcription, for which two rounds of translation (e.g., togavirus) or subgenomic RNAs (e.g., tobamovirus) are necessary to produce the genomic RNA.
- **Class V:** Single-stranded (−)sense RNA.
  As discussed in Chapters 3 and 5, the genomes of these viruses can be divided into two types: (a) nonsegmented genomes (order *Mononegvirales*; Figure 4.18), for which the first step in replication is transcription of

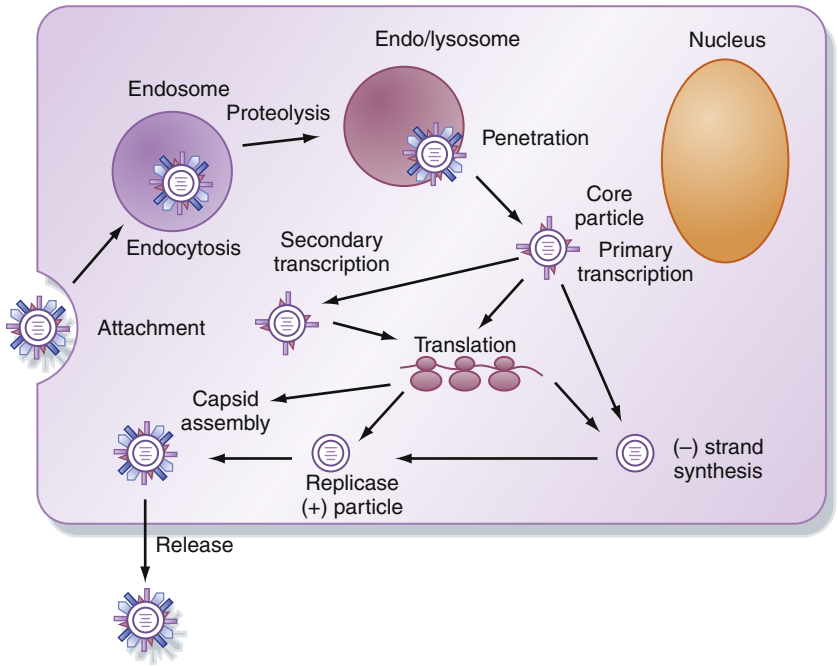

FIGURE 4.16 **Schematic representation of the replication of class III viruses.**
Details of the events that occur for genomes of this type are given in the text.

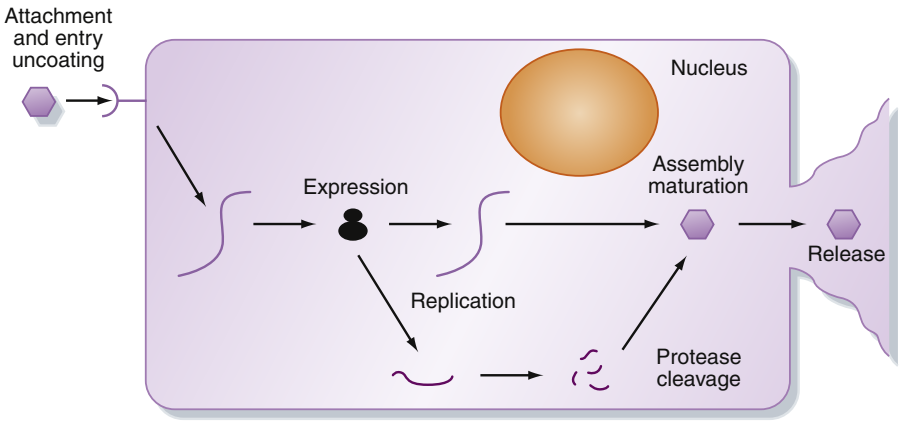

FIGURE 4.17 Schematic representation of the replication of class IV viruses.
Details of the events that occur for genomes of this type are given in the text.

the (−)sense RNA genome by the virion RNA-dependent RNA polymerase to produce monocistronic mRNAs, which also serve as the template for subsequent genome replication (Note: Some of these viruses also have an **ambisense** organization); (b) segmented genomes (*Orthomyxoviridae*), for which replication occurs in the nucleus, with monocistronic mRNAs for each of the virus genes produced by the virus **transcriptase** from the full-length virus genome (see Chapter 5).

- **Class VI:** Single-stranded (+)sense RNA with DNA intermediate (Figure 4.19).
  Retrovirus genomes are (+)sense RNA but unique in that they are diploid and they do not serve directly as mRNA, but as a template for reverse transcription into DNA (see Chapter 3).

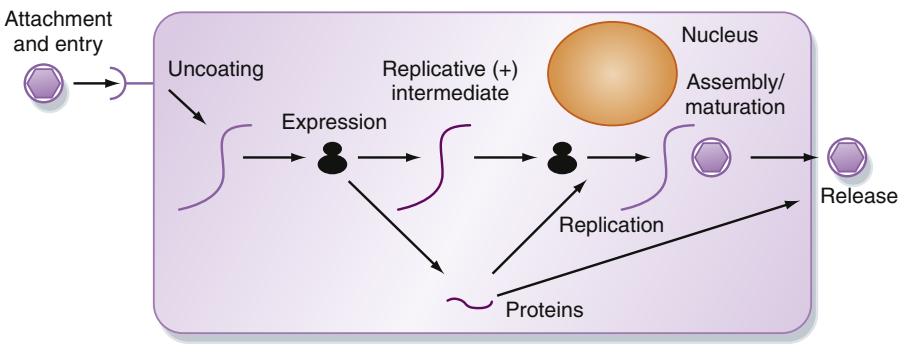

FIGURE 4.18 Schematic representation of the replication of class V viruses.
Details of the events that occur for genomes of this type are given in the text.

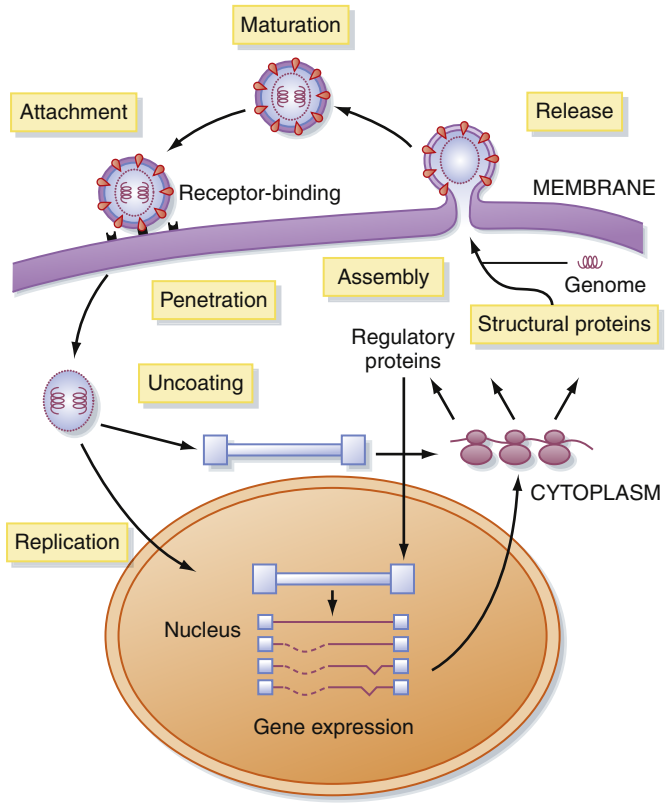

FIGURE 4.19 **Schematic representation of the replication of class VI viruses.**
Details of the events that occur for genomes of this type are given in the text.

- **Class VII:** Double-stranded DNA with RNA intermediate (Figure 4.20). This group of viruses also relies on reverse transcription, but, unlike the retroviruses (class VI), this process occurs inside the virus particle during maturation. On infection of a new cell, the first event to occur is repair of the gapped genome, followed by transcription (see Chapter 3).

Assembly

The **assembly** process involves the collection of all the components necessary for the formation of the mature **virion** at a particular site in the cell. During assembly, the basic structure of the virus particle is formed. The site of assembly depends on the site of replication within the cell and on the mechanism by which the virus is eventually released from the cell and varies for different viruses. For example, in picornaviruses, poxviruses, and reoviruses, assembly occurs in the cytoplasm; in adenoviruses, polyomaviruses, and parvoviruses, it occurs in the nucleus.

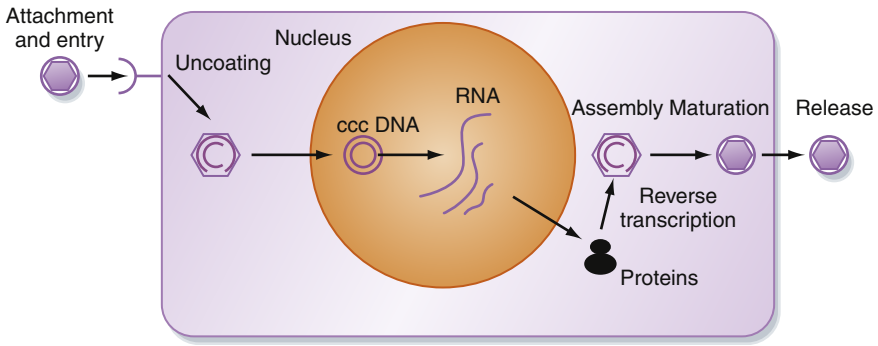

FIGURE 4.20 **Schematic representation of the replication of class VII viruses.**
Details of the events that occur for genomes of this type are given in the text.

Lipid rafts are membrane microdomains enriched with glycosphingolipids (or glycolipids), cholesterol, and a specific set of associated proteins. A high level of saturated hydrocarbon chains in sphingolipids allows cholesterol to be tightly interleaved in these rafts. The lipids in these domains differ from other membrane lipids in having relatively limited lateral diffusion in the membrane, and they can also be physically separated by density centrifugation in the presence of some detergents. Lipid rafts have been implicated in a variety of cellular functions, such as apical sorting of proteins and signal transduction, but they are also used by viruses as platforms for cell entry (e.g., HIV, SV40, and rotavirus), and as sites for particle assembly, budding, and release from the cell membrane (e.g., influenza virus, HIV, measles virus, and rotavirus).

As with the early stages of replication, it is not always possible to identify the assembly, **maturation**, and **release** of virus particles as distinct and separate phases. The site of assembly has a profound influence on all these processes. In the majority of cases, cellular membranes are used to anchor virus proteins, and this initiates the process of assembly. In spite of considerable study, the control of virus assembly is generally not well understood. In general, it is thought that rising intracellular levels of virus proteins and **genome** molecules reach a critical concentration and that this triggers the process. Many viruses achieve high levels of newly synthesized structural components by concentrating these into subcellular compartments, visible in light microscopes, which are known as **inclusion bodies**. These are a common feature of the late stages of infection of cells by many different viruses. The size and location of inclusion bodies in infected cells are often highly characteristic of particular viruses; for example, rabies virus infection results in large perinuclear Negri bodies, first observed using an optical microscope by Adelchi Negri in 1903. Alternatively, local concentrations of virus structural components can be boosted by lateral interactions between

membrane-associated proteins. This mechanism is particularly important in **enveloped** viruses released from the cell by **budding** (see later).

As discussed in Chapter 2, the formation of virus particles may be a relatively simple process that is driven only by interactions between the subunits of the **capsid** and controlled by the rules of symmetry. In other cases, assembly is a highly complex, multistep process involving not only virus structural proteins but also virus-encoded and cellular scaffolding proteins that act as templates to guide the assembly of **virions**. The encapsidation of the virus genome may occur either early in the assembly of the particle (e.g., many viruses with **helical** symmetry are nucleated on the genome) or at a late stage, when the genome is stuffed into an almost completed protein shell.

Maturation

Maturation is the stage of the replication cycle at which the virus becomes infectious. This process usually involves structural changes in the virus particle that may result from specific cleavages of **capsid** proteins to form the mature products or conformational changes that occur in proteins during assembly. Such events frequently lead to substantial structural changes in the capsid that may be detectable by measures such as differences in the antigenicity of incomplete and mature virus particles, which in some cases (e.g., picornaviruses) alters radically. Alternatively, internal structural alterations—for example, the condensation of nucleoproteins with the virus **genome**—often result in such changes. As already stated, for some viruses assembly and maturation occur inside the cell and are inseparable, whereas for others maturation events may occur only after release of the virus particle from the cell. In all cases, the process of maturation prepares the particle for the infection of subsequent cells.

Virus-encoded proteases are frequently involved in maturation, although cellular enzymes or a mixture of virus and cellular enzymes are used in some cases. Clearly there is a danger in relying on cellular proteolytic enzymes in that their relative lack of substrate specificity could easily completely degrade the capsid proteins. In contrast, virus-encoded proteases are usually highly specific for particular amino acid sequences and structures, frequently only cutting one particular peptide bond in a large and complex virus capsid. Moreover, they are often further controlled by being packaged into virus particles during assembly and are only activated when brought into close contact with their target sequence by the conformation of the capsid (e.g., by being placed in a local hydrophobic environment or by changes of pH or metal ion concentration inside the **capsid**).

Retrovirus proteases are good examples of enzymes involved in maturation that are under this tight control. The retrovirus core particle is composed of proteins from the *gag* gene, and the protease is packaged into the core before its **release** from the cell on **budding**. At some stage of the budding process (the exact

timing varies for different retroviruses) the protease cleaves the *gag* protein precursors into the mature products—the capsid, nucleocapsid, and matrix proteins of the mature virus particle (Figure 4.21). Not all protease cleavage events involved in maturation are this tightly regulated. Native influenza virus hemagglutinin undergoes posttranslational modification (glycosylation in the Golgi apparatus) and at this stage exhibits receptor-binding activity. However, the protein must be cleaved into two fragments (HA_1 and HA_2) to be able to produce membrane **fusion** during infection. Cellular trypsin-like enzymes are responsible for this process, which occurs in secretory vesicles as the virus buds

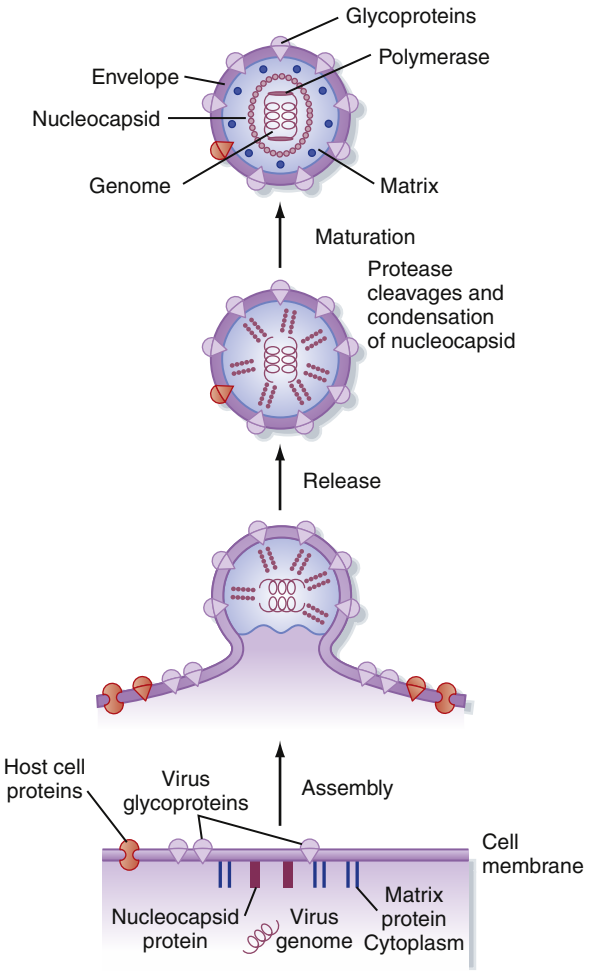

FIGURE 4.21 Virus release by budding.
Budding is the process by which enveloped virus particles acquire their membranes and associated proteins, as well as how they are released for the host cell.

into them prior to **release** at the cell surface. Amantadine and rimantadine are two drugs that are active against influenza A viruses (Chapter 6). The action of these closely related compounds is complex, but they block cellular membrane ion channels. The target for both drugs is the influenza matrix protein (M2), but resistance to the drug may also map to the hemagglutinin gene. The replication of some strains of influenza virus is inhibited at the **penetration** stage and that of others at **maturation**. The biphasic action of these drugs results from the inability of drug-treated cells to lower the pH of the endosomal compartment (a function normally controlled by the M2 gene product), and hence to cleave hemagglutinin during maturation. Similarly, retrovirus **envelope** glycoproteins require cleavage into the surface (SU) and transmembrane (TM) proteins for activity. This process is also carried out by cellular enzymes but in general is poorly understood, but it is a target for inhibitors that may act as antiviral drugs.

Release

As described earlier, plant viruses face particular difficulties caused by the structure of plant cell walls when it comes to leaving cells and infecting others. In response, they have evolved particular strategies to overcome this problem, which are discussed in detail in Chapter 6. All other viruses escape the cell by one of two mechanisms. For **lytic** viruses (such as most nonenveloped viruses), **release** is a simple process—the infected cell breaks open and releases the virus. **Enveloped viruses** acquire their lipid membrane as the virus buds out of the cell through the cell membrane or into an intracellular vesicle prior to subsequent release. **Virion** envelope proteins are picked up during this process as the virus particle is extruded. This process is known as **budding**. Release of virus particles in this way may be highly damaging to the cell (e.g., paramyxoviruses, rhabdoviruses, and togaviruses), or in other cases, appear not to be (e.g., retroviruses), but in either case the process is controlled by the virus. The physical interaction of the **capsid** proteins on the inner surface of the cell membrane forces the particle out through the membrane (Figure 4.15). As mentioned earlier, assembly, maturation, and release are usually simultaneous processes for virus particles formed by budding. The type of membrane from which the virus buds depends on the virus concerned. In most cases, budding involves cytoplasmic membranes (retroviruses, togaviruses, orthomyxoviruses, paramyxoviruses, bunyaviruses, coronaviruses, rhabdoviruses, hepadnaviruses) but in some cases can involve the nuclear membrane (herpesviruses).

In a few cases, notably in human retroviruses such as HIV and HTLV, viruses prefer direct cell-to-cell spread rather than release into the external environment and reuptake by another cell. This process requires intimate contact between cells and can occur at tight junctions between cells or in neurological synapses. These structures have been subverted by human retroviruses that engineer

a novel structure in infected cells known as a virological synapse to promote more efficient spread within the host organism.

The release of mature virus particles from susceptible host cells by budding presents a problem in that these particles are designed to enter, rather than leave, cells. How do these particles manage to leave the cell surface? The details are not known but there are clues as to how the process is achieved. Certain virus **envelope** proteins are involved in the **release** phase of replication as well as in the initiating steps. A good example of this is the neuraminidase protein of influenza virus. In addition to being able to reverse the attachment of virus particles to cells via hemagglutinin, neuraminidase is also believed to be important in preventing the aggregation of influenza virus particles and may well have a role in virus release. This process is targeted by newer drugs such as oseltamivir (trade name Tamiflu) and zanamivir (Relenza; Chapter 6). In recent years, a group of proteins known as viroporins has been discovered in a range of different viruses. These are proteins that modify the permeability of cellular membranes and promote the release of viral particles from infected cells. These proteins are usually not essential for the replication of viruses, but their presence often enhances virus growth.

In addition to using specific proteins, viruses that bud have also solved the problem of release by the careful timing of the assembly—maturation—release pathway. Although it may not be possible to separate these stages by means of biochemical analysis, this does not mean that careful spatial separation of these processes has not evolved as a means to solve this problem. Similarly, although we may not understand all the subtleties of the many conformation changes that occur in virus **capsids** and envelopes during these late stages of replication, virus replication clearly works, despite our lack of knowledge.

BOX 4.3. WORLDS WITHIN WORLDS

We think of eukaryotic cells as compartmentalized into nucleus and cytoplasm, but the true situation is more complicated than that. There are other biochemical rather than physical compartments within a cell. One is the lipid/aqueous division. Proteins with hydrophobic (water-fearing) domains don't like to be in a soluble form within the cytoplasm. They only start to act when they're in the natural environment of a membrane. But it's not even that simple. There are different domains within membranes where different processes occur. Viruses have used these "lipid rafts" for particular functions, such as entering or leaving the cell, and forming tiny factories where new particles are assembled. And then there's time. Virus replication doesn't happen in a random order—it is carefully sequenced to optimize the process. This control is directed by the biochemistry of the components involved, which may only start to function as their concentration within an infected cell reaches a critical level. And all of this goes on within the minute world of an infected cell, too small to see with the eye alone, or even the most powerful microscope.

SUMMARY

In general terms, virus replication involves three broad stages carried out by all types of viruses: the initiation of infection, replication, and expression of the genome, and finally, release of mature virions from the infected cell. At a detailed level, there are many differences in the replication processes of different viruses that are imposed by the biology of the host cell and the nature of the virus genome. Nevertheless, it is possible to derive an overview of virus replication and the common stages which, in one form or another, are followed by all viruses.

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