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Chapter 4

Virus Replication

Understanding the molecular events accompanying virus replication has been a major focus of experimental virology, and is essential for the proper understanding and control of all virus diseases. The biological “purpose” of any replication cycle is the generation of new viral genomes and proteins in sufficient quantities to ensure propagation of the viral genome (a clear example of the “selfish gene”); this requires that the extracellular viral genome is protected from enzymatic degradation and can be introduced into further target cells for further rounds of replication. Much has become known about the initial stages of attachment, and more detailed study shows that the initial recognition between virus and host is more complex than originally supposed. Temporal regulation of intracellular events is critical in all but the very simplest of viruses, with some form of suppression of the host innate immune response being common to nearly all human viruses. There are examples where the innate immune response is even used to enhance virus spread to cells otherwise unavailable to the virus. Over the next few years, the boundaries between virus-directed events and cellular processes that control specialized cell functions are likely to be even more complex; nevertheless understanding these processes will open up a range of targets for the development of novel antiviral therapies and immunotherapy.

This chapter presents an overview of the subject, indicating similarities and differences in the replication strategies adopted by viruses of each family that contains human pathogens. Major features and replication requirements of human viruses are shown in Table 4.1. More detailed information about the replication of individual viruses can be found in the relevant chapters of Part II of this book.

GROWTH OF VIRUSES

Before the development of in vitro culture techniques, viruses of humans could only be propagated in experimental animals. Thus much of our knowledge about virus growth and replication relied in the first instance on the study of bacterial viruses.

In 1931 it was shown that vaccinia virus and herpes simplex virus could be grown on the chorioallantoic membrane of embryonated chicken eggs. This system became routine for the study of many mammalian viruses as virtually all virus families contain viruses that can be cultured in this way. Although cell culture has long since replaced the use of eggs for this purpose, the technology is still in use for the preparation of influenza virus stocks and the manufacture of influenza vaccines.

The development of in vitro cell culture systems was a watershed development in virology: not only did it become possible to dissect the intracellular events accompanying virus replication in a manner similar to that of the study of bacteriophages in bacterial cells, it also provided a means of quantifying the amount of infectious virus in samples and virus stocks. Artificial medium was developed to maintain cell viability independent of the source species: these cells could be in the form of organ cultures, explant cultures, primary cell culture monolayers, or monolayer cell cultures immortalized into cell lines. Organ cultures maintain the three-dimensional structure of the tissue of origin and can be useful for short-term experiments that depend upon preserving fully differentiated cells. For example, tracheal epithelial cells attached to the cartilage matrix of the trachea during culture played a critical role in the isolation of many human respiratory viruses. The preparation of primary cell cultures uses proteases such as trypsin or collagenase to separate individual cells of a tissue such as fetal kidney or lung, and the individual cells then attach to a cell culture substrate where a limited number of divisions will occur. The limited life span of these cells requires repeated preparation of new cultures from source tissue, clearly presenting problems with reproducibility. In contrast, continuous propagation of cells is possible with two types of long-term culture:

1. “Semicontinuous,” “diploid” cell strains, for example, human lung or foreskin fibroblasts (WI-38, MRC-5), in which cells eventually senesce after 20 to 30 divisions (the “Hayflick number”) due to progressive shortening of telomeres, and

2. Continuous immortalized cell lines, for example, HeLa cells, BHK-21 cells. These are derived either from tumors or from primary cells that have undergone a spontaneous transformation event during cell culture, and can undergo an almost infinite number of cell divisions, thus generating consistency although often accompanied by a loss of differentiated cell functions. For example, HeLa cells were originally isolated in cell culture from a patient who died of cervical cancer.
in 1951, but the cells remain an important system for culturing viruses to this day, and in fact proliferate so successfully that HeLa cells can become contaminants of cell cultures from other sources.

Recognition of the presence of a virus was dependent initially on observing cultures at regular intervals under the light microscope for signs of morphological change or cell death, compared to uninoculated cell cultures acting as controls. The specific appearance of the cytopathic effect (cpe) is often diagnostic for a certain family of viruses, for example herpesviruses can cause a distinct cytopathology often accompanied by the fusion of dying cells. However, reliance on cytopathology can give false results especially

| Family            | Route of Uptake                              | Site of Nucleic Acid Replication | Eclipse Period (h) | Site of Maturation or Budding |
|-------------------|---------------------------------------------|----------------------------------|--------------------|-------------------------------|
| Poxviridae        | Variable                                    | Cytoplasm                        | 4                  | Golgi membrane                |
| Herpesviridae     | Variable                                    | Nucleus                          | 4                  | Nuclear membrane              |
| Adenoviridae      | Clathrin-mediated endocytosis               | Nucleus                          | 10                 | Nucleus                       |
| Papillomaviridae  | Clathrin-mediated endocytosis               | Nucleus                          | †                  | Nucleus                       |
| Polyomaviridae    | Caveolar endocytosis                        | Nucleus                          | 12                 | Nucleus                       |
| Paroviridae       | Clathrin-mediated endocytosis               | Nucleus                          | 6                  | Nucleus                       |
| Circoviridae      | ?                                           | Nucleus                          | †                  | None                          |
| Hepadnavirida     | Clathrin-mediated endocytosis               | Nucleus and cytoplasm            | †                  | Endoplasmic reticulum         |
| Retroviridae      | Plasma membrane fusion or clathrin-mediated endocytosis | Cytoplasm and nuclear membrane | 10                 | Plasma membrane               |
| Reoviridae        | Clathrin-mediated endocytosis               | Cytoplasm                        | 5                  | Cytoplasm                     |
| Birnaviridae      | Uncertain                                   | Cytoplasm                        | 4                  | None                          |
| Paramyxoviridae   | Plasma membrane fusion                      | Cytoplasm                        | 4                  | Plasma membrane               |
| Rhabdoviridae     | Plasma membrane fusion                      | Cytoplasm                        | 3                  | Plasma membrane               |
| Filoviridae       | Plasma membrane fusion                      | Cytoplasm                        | 2                  | Plasma membrane               |
| Bornaviridae      | Clathrin-mediated endocytosis               | Nucleus                          | †                  | Plasma membrane               |
| Orthomyxoviridae  | Clathrin-mediated endocytosis               | Nucleus                          | 4                  | Plasma membrane               |
| Bunyaviridae      | Clathrin-mediated endocytosis               | Cytoplasm                        | 4                  | Golgi membrane                |
| Arenaviridae      | Clathrin-mediated endocytosis               | Cytoplasm                        | 5                  | Plasma membrane               |
| Coronavirus        | Clathrin-mediated endocytosis / plasma membrane fusion | Cytoplasm                        | 5                  | Endoplasmic reticulum         |
| Arteriviridae     | Clathrin-mediated endocytosis               | Cytoplasm                        | 5                  | Endoplasmic reticulum         |
| Picornaviridae    | Caveolar endocytosis / plasma membrane insertion | Cytoplasm                        | 2                  | Cytoplasm                     |
| Caliciviridae     | Caveolar endocytosis / plasma membrane insertion | Cytoplasm                        | 3                  | Cytoplasm                     |
| Astroviridae      | Caveolar endocytosis / plasma membrane insertion | Cytoplasm                        | 3                  | Cytoplasm                     |
| Togaviridae       | Clathrin-mediated endocytosis               | Cytoplasm                        | 2                  | Plasma membrane               |
| Flaviviridae      | Clathrin-mediated endocytosis               | Cytoplasm                        | 3                  | Endoplasmic reticulum         |

*Differs with multiplicity of infection, strain of virus, cell type, and physiological conditions.*
if the sample is contaminated with bacterial toxins or other adventitious agents not related to the pathological process under study.

Using such systems, the basic mechanisms of transcription, translation, and nucleic acid replication have been characterized for all the major families of vertebrate viruses and the strategies of gene expression and regulation have been clarified. Every family of viruses employs unique replication strategies. One important unifying and simplifying concept, as originally proposed by David Baltimore in 1971, was to assign all viruses to one of six classes based on their genome composition and the pathway used to produce mRNAs for translation using host cell ribosomes (Fig. 4.1). This also reflects the parasitic property of all viruses in requiring a host cell for the synthesis of viral proteins.

**THE VIRUS REPLICATION CYCLE**

Most studies of the replication of viruses of humans have used cultured mammalian cells, grown either in suspension or as a monolayer adherent to a plastic surface. Classic studies of this kind defined the one-step growth curve, in which all cells in a culture are infected simultaneously by using a high multiplicity of infection, and the increase in infectious virus over time is measured by sequential sampling and titration of infectious virus (Fig. 4.2). It is important to understand that, with synchronous infection of the whole cell population, the events observed in the total culture can be used to study events in a single cell. The time to achieve maximal yield of virus ranges from 6 hours (poliovirus) to more than 24 hours (adenovirus), compared with a generation time for *E. coli* in broth culture of 15 to 20 minutes.

Virus released into the medium can be titrated separately from virus that remains cell-associated. Shortly after infection, the inoculated virus “disappears”—infectious particles cannot be detected, even if the cells are disrupted. This eclipse period continues until the first progeny virions are detected some hours later. As infection progresses, non-enveloped viruses mature within the cell and may be detected as infectious, intracellular virions for some hours before being released by cell lysis. Many enveloped viruses, on the other hand, mature by budding from the plasma membrane.
Virus release via cell lysis is abrupt, whereas release via budding may continue over a protracted period of time. The eclipse period generally ranges from 2 to 12 hours for viruses of different families.

Early studies, relying on quantitative electron microscopy and the assay of infectious virions, provided an overview of the replication cycle (attachment, penetration, maturation, and release), but with little detail as to the processes involved. Investigation of the expression and replication of the viral genome became possible after the introduction of biochemical methods for the analysis of viral nucleic acids and proteins. Furthermore, imaging methods are also available nowadays that enable the tracking of viral and cellular proteins as the viral replication cycle proceeds.

**Attachment**

In order to initiate infection, virions must first bind to cells. Binding occurs between ligands on the surface of the virion (viral attachment proteins) and receptors on the plasma membrane of the cell (Table 4.2). This initial interaction between virus and host cell is sometimes complex and there is frequently a lack of correlation between attachment studies in cultured cells versus intact hosts. For example, several viral envelope glycoproteins of herpesviruses may serve as attachment proteins and several cellular receptors may be involved, in sequential order, first achieving a loose attachment via one receptor, then irreversibly binding via a second receptor and ligand. Exploitation of more than one cellular ligand provides redundancy and also may assist herpesviruses in invading both epithelial and neural tissues to support latent/recrudescent infection cycles.

While there is a degree of specificity about the recognition of particular cellular receptors by particular viruses, quite different viruses (e.g., orthomyxoviruses and paramyxoviruses) may utilize the same receptor and, conversely, viruses in the same family or genus may use different receptors. Viruses have thus evolved by making opportunistic use of a wide variety of host cell surface proteins as receptors—in most cases viruses employ as receptors host cell proteins that are crucial for fundamental cellular functions, many of which are strongly conserved over evolutionary time.

The receptor–ligand interaction between human cells and human immunodeficiency virus 1 (HIV-1) illustrates the complexity of the interaction between virus and host proteins. Attachment initially involves CD4 molecules on the surface of target cells, notably macrophages and T helper lymphocytes, via the viral gp120 envelope glycoprotein. This binding induces a conformational change exposing a high-affinity chemokine receptor-binding site on gp120, which binds one of several chemokine receptors on the cell surface. This latter binding in turn exposes a fusogenic domain of the viral transmembrane protein gp41. Direct contact between the fusogenic domain of gp41 and the cell membrane brings about the fusion of the viral envelope with the plasma membrane of the cell, permitting the viral nucleocapsid to enter the cell cytoplasm (see Fig. 23.6).

The cellular receptor for most orthomyxoviruses is the terminal sialic acid on an oligosaccharide side-chain of a glycoprotein (or glycolipid) exposed at the cell surface: the viral ligand is in a cleft at the distal tip of each monomer of the trimeric viral hemagglutinin glycoprotein (see Chapter 3: Virion Structure and Composition). Viruses can adapt to new hosts through modification of the relevant attachment ligand. For example, influenza A viruses originate in aquatic birds and recognize sialic acid residues on the surface of cells along the respiratory tract. However, influenza A viruses of birds have a greater affinity for sialic acid linked to the penultimate galactose moiety through α2–3 bonding, whereas those of humans have a greater affinity for sialic
acid linked via an $\alpha_2$–6 bond; this reflects differences in the relative abundance of the two linkages in the different host species. There is considerable subtlety in these interactions, and as with many human and animal viruses a single amino acid change within a receptor site may manifest as a significant change in host range and epidemiology.

Proinflammatory reactions in the host may also affect the expression of receptors and thereby indirectly modify interactions between virus and potential host cells. For example, human adenovirus 5 recognizes both the Coxsackievirus and adenovirus receptor (CAR) and $\alpha_v\beta_3$ integrin in order to infect cells, yet neither is normally exposed on the apical surfaces of cells until macrophages respond to infection and secrete IL-8. This in turn moves the CAR and $\alpha_v\beta_3$ integrin receptors away from the tight junctions of polarized cells toward the apical cell surface and thus become exposed and available for virus attachment.

### Penetration and Uncoating

The majority of mammalian cells are continuously engaged in receptor-mediated endocytosis for the uptake of macromolecules via specific receptors. Many enveloped and non-enveloped viruses use this essential cell function to initiate penetration and uncoating (Fig. 4.3). Virion attachment to those receptors clustered at clathrin-coated pits is followed by endocytosis into clathrin-coated vesicles. Vesicles enter the cytoplasm and, after removal of the clathrin coat, fuse with endosomes (acidic prelysosomal vacuoles). Acidification within the vesicle triggers changes in virion proteins and surface structures. For example, the acidic pH within the endosome, induces the hemagglutinin molecules of influenza viruses to undergo a conformational change. This enables fusion to occur between the viral envelope and the endosomal membrane and results in the release of the viral nucleocapsid into the cytoplasm. Many other non-enveloped and enveloped viruses undergo comparable changes.

Fusion at neutral pH is an alternative mechanism. The F (fusion) glycoprotein of paramyxoviruses causes the viral envelope to fuse directly with the plasma membrane of the cell, even at pH 7. This allows the nucleocapsid to be released directly into the cytoplasm. A number of other important enveloped viruses for example filoviruses, have

### Table 4.2 Examples of Cellular Receptors for Viruses of Medical Importance

| Virus                     | Family         | Receptor                                                                 |
|---------------------------|----------------|--------------------------------------------------------------------------|
| Human immunodeficiency    | Retroviridae   | CD4; coreceptors CCR5, CXCR4, CCR3                                        |
| Vaccinia virus            | Poxviridae     | Various (heparan sulfate, chondroitin sulfate glycosaminoglycans, laminin) |
| Poliovirus                | Picornaviridae | PVR (CD155)—Immunoglobulin (Ig) superfamily                             |
| Human rhinovirus 14       | Picornaviridae | Intercellular adhesion molecule-1 (ICAM-1); Ig superfamily              |
| Echovirus 1               | Picornaviridae | $\alpha_2/\beta_1$ integrin VLA-2                                         |
| Hepatitis A virus         | Picornaviridae | HAVCR1; member of TIM-1 family (transmembrane, Ig, mucin)                |
| Adenovirus                | Adenoviridae   | CAR (Coxsackie and adenovirus receptor), member Ig superfamily; coreceptor $\alpha_v$, integrins |
| Herpes simplex virus 1    | Herpesviridae  | HveA (herpesvirus entry mediator A), heparan sulfate proteoglycan       |
| Human cytomegalovirus     | Herpesviridae  | Heparan sulfate proteoglycan                                             |
| Epstein–Barr virus        | Herpesviridae  | CD21, complement receptor 2 (CR2)                                         |
| Adeno-associated virus 5   | Parvoviridae   | $\alpha(2,3)$-linked sialic acid                                         |
| Influenza A virus          | Orthomyxoviridae | $\alpha(2,3)$- or $\alpha(2,6)$-linked sialic acid                      |
| Influenza C virus          | Orthomyxoviridae | 9-O-acetylsialic acid                                                      |
| Hendra virus               | Paramyxoviridae | Ephrin-B2                                                                |
| Rotavirus                  | Reoviridae     | Various integrins                                                        |
| Reovirus                   | Reoviridae     | JAMs (junction adhesion molecules)                                       |
| Dengue virus               | Flaviviridae   | Heparan sulfate proteoglycan                                             |
| Rabies virus               | Rhabdoviridae  | Acetylcholine, NCAM (neural adhesion molecule)                           |
| Polyomaviruses             | Polyomaviridae | Various gangliosides (glycosphingolipids with terminal sialic acid)      |
the ability to fuse with the host cell plasma membrane, thereby allowing entry of viral nucleic acid into the cytosol.

A third entry mechanism ("direct entry") is used by some non-enveloped viruses (e.g., poliovirus, adenovirus). This involves binding of virus to receptors at the endosomal membrane, which induces conformational changes in the viral capsid; these changes then expose regions that react with the endosomal membrane to induce channels for transport of the genome across the plasma membrane (Fig. 4.4).

**FIGURE 4.3** Endocytic mechanisms for virus entry. Endocytosis in animal cells can occur via four major mechanisms: clathrin-mediated endocytosis (CME); macropinocytosis; caveolae/lipid rafts; and phagocytosis (larger particles, >0.75 μM). Note the IL-2, GEEC (GPI-enriched early endosomal compartment), Flotillin and Arf6 (ADP-ribosylation factor 6) pathways import specific cell cargo but viruses are not currently known to use these routes. VSV, vesicular stomatitis virus; SFV, Semliki Forest virus; HSV, herpes simplex virus; LCMV, lymphocytic choriomeningitis virus; SV40, simian virus 40; mPy, mouse polyomavirus; HPV, human papillomavirus. Reproduced from MacLachlan, N.J., Dubovi, E.J., 2011. Veterinary Virology, fourth ed. Academic Press, London (Fig. 2.5), with permission.

**FIGURE 4.4** Mechanism of poliovirus entry. Virus binds to specific receptors on host cell plasma membrane (CD155 = poliovirus receptor Pvr). The receptors induce conformational changes in the virion (the 160S infectious virion becomes a 135S particle), with the loss of VP4 and the externalization of the N-terminus of VP1. The 135S particles are then internalized by a clathrin- and caveolin-independent, but actin- and tyrosine kinase-dependent, mechanism. The release of the viral genome takes place only after internalization from an endocytic compartment localized within 100 to 200 nm of the plasma membrane. Upon release of the RNA genome, the empty capsid (80S) is transported away along microtubules. Reproduced from Brandenburg, B., et al., 2007, Imaging poliovirus entry in live cells. PLoS Biol 5 (7), e183. doi:10.1371/journal.pbio.0050183, with permission.
In order for viral genes to become available for transcription, it is necessary that virions are at least partially uncoated. In the case of enveloped RNA viruses entering by the process of fusion, the nucleocapsid is discharged directly into the cytoplasm and transcription commences from viral nucleic acid while still associated with the nucleocapsid protein(s). With the non-enveloped icosahedral reoviruses, only certain capsid proteins are removed and the viral genome expresses all its functions without being released from the virion core. For most other viruses, however, uncoating proceeds to completion, otherwise genome duplication cannot proceed. For some viruses, uncoating takes place in the nucleus where later stages of replication occur.

**Replication of Viral Nucleic Acids**

**Replication of Viral DNA**

Different mechanisms of DNA replication are employed by each family of DNA viruses (Fig. 4.5). These involve either synthesis of daughter strands via a replication fork (papillomaviruses, polyomaviruses, herpesviruses), or via strand displacement (adenoviruses, paroviruses, poxviruses). Since cellular DNA polymerases cannot initiate synthesis of a new DNA strand but can only extend synthesis beginning from a short (e.g., RNA) primer, one end of newly synthesized viral DNA molecules might be expected to retain a short single-stranded region. Various DNA viruses have evolved different strategies for circumventing this problem. Viruses of some families have a circular DNA genome, others have a linear genome with complementary termini that serve as DNA primers, yet others have a protein primer covalently attached to the 5‘-terminus of each DNA strand.

Several virus-coded enzyme activities are generally required for replication of viral DNA: a helicase (with ATPase activity) to unwind the double helix; a helix-stabilizing protein to keep the two separated strands apart until each has been copied; a DNA polymerase to copy each strand from the origin of replication in a 5′- to 3′-direction; an RNAase to degrade the RNA primer after it has served its purpose; and a DNA ligase to join the Okazaki fragments together. Often a single large enzyme performs two or more of these activities.

The genomes of papillomaviruses and polyomaviruses structurally and functionally resemble cellular DNA in binding to cellular histones. The viral genome utilizes host DNA polymerase α-primase to synthesize the RNA primer for genome replication. Among the polyomaviruses an early viral antigen, large T, binds to the regulatory sequence—and in the case of papillomaviruses E1 and E2—thereby initiating DNA replication. Replication of these circular double-stranded DNA molecules commences from a unique palindromic sequence and proceeds simultaneously with replication forks in both directions. Both continuous and discontinuous DNA synthesis occur (of leading and lagging strands respectively) at the two growing forks as for the replication of mammalian DNA. The discontinuous synthesis of the lagging strand involves repeated synthesis of short oligoribonucleotide primers, which in turn initiate short nascent strands of DNA (Okazaki fragments), each is then covalently joined by a DNA ligase to form one of the growing strands.

The replication of adenoviruses is distinct from that of other DNA viruses. The adenovirus DNA genome is linear, the 5′-end of each strand being identical to the other (terminally repeated inverted sequences) and covalently linked to a protein, the precursor of which serves as the
primer for viral DNA synthesis. DNA replication proceeds from both ends, continuously but asynchronously, in a 5′- to 3′-direction, using a virus-coded DNA polymerase. It does not require the synthesis of Okazaki fragments.

Herpesviruses code for many or all of the proteins required for DNA replication, including a DNA polymerase, a helicase, a primase, a single-stranded DNA binding protein, and a protein recognizing the origin of replication. Poxviruses, which replicate entirely within the cytoplasm, are self-sufficient in DNA replication machinery. Hepadnaviruses, similar to retroviruses, utilize positive-sense single-stranded RNA transcripts as intermediates for the production of progeny DNA by a process of reverse transcription. Single-stranded DNA paroviruses use 3′-palindromic sequences to form a double-stranded hairpin structure to provide a primer for cellular DNA polymerase binding.

**Replication of Viral RNA**

Transcription of RNA from an RNA template is a phenomenon unique to viruses (Fig. 4.6), and requires an RNA-dependent RNA polymerase, a virus-coded enzyme not found in uninfected cells. The replication of viral RNA requires first the synthesis of complementary RNA: this in turn serves as a template for the synthesis of further copies of viral RNA.

For viruses where the viral RNA is of negative-sense (orthomyxoviruses, paramyxoviruses, rhabdoviruses, filoviruses, bornavirus, arenaviruses, and bunyaviruses), the complementary RNA is of positive-sense and the RNA polymerase involved performs the same function as the virion-associated transcriptase used for the primary transcription of mRNAs. Most transcripts from such

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**FIGURE 4.6** Strategies for replication and mRNA synthesis of RNA virus genomes are shown for representative virus families. Picornaviral genomic RNA is linked to VPg at the 5′-end. The (+) genomic RNA of some flaviviruses does not contain poly(A). Only one RNA segment is shown for segmented (+) strand RNA viruses. Cap structures are shown as blue boxes containing “C”. Reproduced from Flint, S.J., et al., 2016. Principles of Virology, 4th ed. Washington, DC, ASM Press, with permission.
negative-sense viral RNAs are subgenomic mRNA molecules, but some full-length positive-sense strands must also be made, in order to serve as templates for full-length progeny viral RNA synthesis (replication). For some viruses there is good evidence that the RNA polymerases used for transcription and replication are distinct, in others the same enzyme performs both functions.

In the case of the positive-sense RNA viruses (picornaviruses, caliciviruses, togaviruses, flaviviruses, coronavirus, and arteriviruses), the complementary RNA is negative-sense, and its sole purpose is to provide a template for synthesis of more positive-sense RNA. Several viral RNA molecules can be transcribed simultaneously from a single complementary RNA template, each RNA transcript being the product of a separately bound polymerase molecule. The resulting structure, known as the replicative intermediate, is therefore partially double-stranded with single-stranded tails. Some RNA-dependent RNA polymerases can initiate RNA synthesis de novo, while others require a primer with a free 3′-OH group to which further nucleotides can be added. Initiation of the replication of picornavirus and calicivirus RNA, similar to that of adenovirus DNA, requires a bound protein as primer, rather than an oligonucleotide. This small protein is covalently attached to the 5′-terminus of nascent positive and negative RNA strands in addition to virion RNA, but not to molecules used as mRNA. Little is known about what determines whether a given picornivirus positive-sense RNA molecule will be directed (1) to a replication complex (a structure bound to smooth endoplasmic reticulum), where it serves as template for transcription by RNA-dependent RNA polymerase into negative-sense RNA; or (2) to a ribosome, where it serves as mRNA for translation into protein; or (3) to a procapsid, with which it associates to form a virion.

Other RNA polymerases require an oligonucleotidyl 5′ cap structure, which may be synthesized by viral enzymes or derived from cellular mRNAs by a process of “capping-snatching” (e.g., Chapter 25: Orthomyxoviruses).

Retroviruses have a genome consisting of positive-sense single-stranded RNA. Unlike other RNA viruses, retroviruses replicate via a DNA intermediate. The virion-associated reverse transcriptase, using a transfer RNA (tRNA) molecule as a primer, makes a single-stranded DNA copy, while the same enzyme functions concurrently as a ribonuclease and removes the parental RNA molecule from most of the DNA–RNA hybrid, except for several short RNA stretches known as poly-purine tracts. The poly-purine tracts then act as primers for copying the negative-sense single-stranded DNA strand to form a linear double-stranded DNA that contains an additional sequence known as the long terminal repeat (LTR) at each end. This double-stranded DNA then is processed by the viral integrase and integrates into cellular chromosomal DNA. From this point on, transcription of viral RNA occurs from the integrated (proviral) DNA (see Chapter 23: Retroviruses).

### Overview of Viral Gene Expression Strategies

Distinguishing the various strategies used by viruses for the production of viral proteins is fundamental to an understanding of virus replication (Fig. 4.1). A summary of the properties of viral proteins is given in Table 4.3. For many viruses, the production of viral proteins immediately after entry of the viral genome is a critical step. These early proteins act to subvert the molecular machinery of the host,

| Protein Category | Examples and Comments |
|------------------|-----------------------|
| Structural proteins of the virion | 1. Capsid, and (for some viruses) core and/or envelope and matrix proteins |
| Non-structural proteins, mainly enzymes, required for transcription, replication of viral nucleic acid, and cleavage of proteins | 2. Virion-associated enzymes, especially polymerases (transcriptases) |
| Regulatory proteins which control the temporal sequence of expression of the viral genome | DNA and RNA polymerases, helicases, proteases, etc. DNA viruses with large complex genomes, notably poxviruses and herpesviruses, also encode numerous enzymes needed for nucleotide synthesis |
| Proteins down-regulating expression of cellular genes | Usually by inhibiting transcription, sometimes translation |
| Oncogene products (oncoproteins) and inactivators of cellular tumor suppressor proteins | Upgrade expression of certain cellular genes; may lead to cell transformation and eventually to cancer. Observed with herpesviruses, adenoviruses, papovaviruses, and retroviruses |
| Proteins influencing viral virulence, host range, tissue tropism, etc. | Recorded so far mainly in the more complex DNA viruses (poxviruses, herpesviruses, adenoviruses) but may be more widespread |

**Virokines,** which act on non-infected cells to modulate the progress of infection in the body as a whole, are mainly by subverting the immune response, e.g., by inhibiting cytokines, downregulating MHC expression, blocking the complement cascade, etc.

*RNA viruses of positive-sense and DNA viruses that replicate in the nucleus do not carry a transcriptase in the virion. Virions of some viruses, e.g., poxviruses, also contain many other enzymes.*
to allow the later production of new virus particles and to inhibit the activation of the host innate immunity that would otherwise prevent virus replication and spread to adjacent cells and tissues.

For most families of DNA viruses, transcription and DNA replication take place in the cell nucleus, using cellular RNA polymerase II and other cellular enzymes. For viruses such as poxviruses and herpesviruses it is possible to identify a significant number of genes by gene deletion (more than 40%) that are not essential for the replication of the virus in cultured cells. Of course, in the strict economy of viral genomes it is likely that most or all of such genes are important for virus survival in nature.

The situation is quite different for RNA viruses as these are unique having genetic information coded as RNA. Thus an effort needs to be made to understand the distinct replication and expression strategies, particularly as these have a bearing on understanding virus pathogenesis. RNA viruses with different types of genomes (single-stranded or double-stranded, positive- or negative-sense, monopartite or segmented) have necessarily evolved different routes to the production of mRNA. Since eukaryotic cells do not contain an RNA-dependent RNA polymerase, all RNA viruses need to code for a unique RNA-dependent RNA polymerase. In the case of positive-sense single-stranded RNA viruses, the incoming RNA genome can bind directly to ribosomes and be translated in full or in part without the need for any prior transcription; all other forms of incoming viral RNA must first be transcribed to produce mRNA, in order to begin the process of expression of the infecting viral genome. Thus, both negative-sense single-stranded RNA viruses and double-stranded RNA viruses need to carry an RNA-dependent RNA polymerase in the virion, originating from the preceding round of infection.

In the case of DNA viruses dependent upon the nucleus for replication, cellular DNA-dependent RNA polymerase II performs this function, while double-stranded DNA viruses that replicate in the cytoplasm carry a virus-encoded DNA-dependent RNA polymerase.

Many, but not all, viruses express different genes at different stages of the replication cycle. Early viral genes are first transcribed into RNA, which may then be processed in a number of ways, including splicing. The early gene-products translated from this mRNA are of three main types: proteins that shut down cellular nucleic acid and protein synthesis, proteins that regulate the expression of the viral genome, and enzymes required for the replication of the viral nucleic acid. Following viral nucleic acid replication, late viral genes are transcribed.

The late proteins are principally viral structural proteins used for assembly of new virions; some of these are subject to post-translational modifications before use, and are often made in considerable excess. Structural proteins for the coating of nascent viral genomes are required in multiple copies for every new nucleic acid molecule destined for encapsidation. For this reason, many viral strategies have evolved so that new copies of the viral genome can act as templates for the further specific transcription and translation of structural proteins required for new virus particles.

The existence of overlapping reading frames, multiple splicing patterns of RNA transcripts, post-translational cleavage of polyproteins, etc., mean that it is too simplistic to assume one particular gene codes for a particular protein. It is more appropriate to refer to a transcription unit, defined as a region of the genome beginning with the transcription initiation site and extending to the transcription termination site (including all introns and exons in between), the expression of which falls under the control of a particular promoter.

Some viral proteins serve as regulatory proteins, modulating the transcription or translation of cellular genes or down-regulating early viral genes. The large DNA viruses also encode numerous additional proteins, called virokines, which do not regulate the viral replication cycle per se, but influence the host response to infection. Included among these are viral protein homologues of cellular cytokines.

In 1978 Walter Fiers and his colleagues presented the first complete description of the genome of an animal virus, namely that of the polyomavirus SV40 (see Fig. 20.2). Analysis of the circular double-stranded DNA molecule and its transcription revealed some remarkable insights, many of which are now applicable to other double-stranded DNA viruses. First, early and late genes are transcribed in opposite directions, from different strands of the DNA. Second, certain genes overlap, the protein products generated having common amino acid sequences. Third, some regions of the viral DNA may be read in different reading frames (ORFs), so that quite distinct amino acid sequences are translated from the same nucleotide sequence. Fourth, certain long stretches of the viral DNA consist of transcribed introns that are not translated into protein as these are spliced out of the primary RNA transcript (see Fig. 20.2).

Adenoviruses exemplify some of the mechanisms that regulate the expression of viral genomes at the level of transcription. There are several adenovirus transcription units; at different stages of the viral replication cycle, “pre-early,” “early,” “intermediate,” and “late” transcription units are transcribed in a precise temporal sequence. A product of the early region E1A induces transcription from the other early regions including E1B, but following viral DNA replication there is a 50-fold increase in the rate of transcription from the major late promoter relative to early promoters such as E1B, and a decrease in E1A mRNA levels. A second control operates at the point of termination of transcription. Early in the replication cycle transcripts terminate at a particular point in the genome, while later in infection these are read through the point of termination.
to produce a range of longer transcripts with different polyadenylation sites and proteins of different functions. This is but one of the many examples of the economy of viral genomes in coding for complex functions using minimal sequences of nucleic acid.

For RNA viruses, the regulation of transcription is, on the whole, not as complex as is the case for DNA viruses. In particular, the temporal separation into early genes transcribed before the replication of viral nucleic acid, and late genes thereafter, is not nearly so well defined. In the simplest examples (e.g., picornaviruses), a full-length polycistronic mRNA is translated and the resulting polyprotein is subsequently cleaved to yield equimolar amounts of all protein products. Togaviruses synthesize excess amounts of structural proteins from a separate subgenomic RNA.

Yet other mechanisms of regulation have evolved among viruses with non-segmented negative-sense RNA genomes. Once the nucleocapsid is released into the cytoplasm of an infected cell, the RNA polymerase initiates transcription from the 3’-end of the genome. With paramyxoviruses, discrete genes along the viral RNA are each separated by a consensus sequence that includes termination and start signals as well as short intergenic sequences of U residues enabling the transcriptase to generate a long poly(A) tail for each mRNA by a process of reiterated copying (also known as stuttering). Each discrete mRNA is released from the template while the enzyme continues to transcribe the next gene in sequence. As the polymerase moves from 3’ to 5’ along the template, decreasing amounts of each mRNA are sometimes made due to decreasing efficiency of the transcription process—thus, gene order becomes an efficient way of modulating the relative amount of each protein synthesized.

Paramyxovirus transcription also involves a process known as RNA editing. The P gene codes for two proteins, P and V, which share a common N-terminal amino acid sequence but differ completely in the C-terminal sequences as a result of a shift in the reading frame brought about by the insertion of two uncoded G residues into the RNA transcript by transcriptase stuttering.

Eukaryotic cells are not equipped to translate polycistronic mRNA into several individual species of protein, as mammalian cells cannot normally reinitiate translation partway along an RNA molecule. DNA viruses overcome this limitation by using cellular mechanisms for cleavage (and sometimes splicing) of viral polycistronic RNA transcripts to yield monocistronic mRNA molecules.

RNA viruses, most of which replicate in the cytoplasm, do not have access to the RNA processing and splicing enzymes of the host cell nucleus, but have developed a remarkable diversity of solutions to the difficulty of punctuating a large genome to produce multiple individual gene products. Some (e.g., orthomyxoviruses) have developed a segmented RNA genome in which each gene is, in general, expressed and duplicated as a separate molecule. Others (e.g., paramyxoviruses) have evolved a polycistronic genome but produce monocistronic RNA transcripts by termination and reinitiation of transcription. Yet other RNA viruses (e.g., coronaviruses) make use of a nested set of overlapping RNA transcripts, each of which is translated into a single gene product. Finally, some (e.g., picornaviruses) have a polycistronic genomic RNA, which is translated into a polyprotein that is later cleaved proteolytically to yield the final products.

With certain viruses, polycistronic mRNA can be translated directly to produce several gene-products as a result of initiation, or reinitiation, of translation at internal AUG start codons. Internal initiation of translation is facilitated by an upstream RNA motif known as an internal ribosomal entry site (IRES), first discovered in 1988 in poliovirus and encephalomyocarditis virus RNAs (another picornavirus). Where initiation of translation at an internal AUG takes place, a frameshift can also occur. Another mechanism, known as ribosomal frameshifting, occurs fortuitously when a ribosome happens to slip one nucleotide back and forth along an RNA template. This phenomenon is exploited by retroviruses, where frameshift read through leads to about 5% of Gag protein molecules being extended as a Gag-Pol polyprotein. Thus, taken together with the phenomenon of RNA splicing and RNA editing described above, it can be seen that there are several mechanisms of exploiting overlapping reading frames to maximize the limited coding potential of viruses with comparatively small genomes.

**Regulatory Genes and Post-transcriptional Processing**

There is considerable interest in the untranslated regions of viral genomes, particularly the numerous conserved (consensus) sequences or motifs that represent responsive elements. Many of the latter have a critical role to play in the regulation of transcription. For example, each transcription unit of a viral genome has near its 3’-end an mRNA transcription initiation site (start site), designated as nucleotide +1. Within the hundred or so nucleotides upstream of the start site is the promoter, which upregulates the transcription of a particular gene or series of genes. Upstream or downstream from the start site there may be a long sequence with several, in some cases repeated, elements known as enhancers that amplify transcription even further. These regulatory regions are activated by the binding of either viral or cellular DNA-reactive proteins. Several such proteins may bind to adjacent responsive elements to form an interactive structure, or otherwise interact to facilitate attachment of the viral RNA polymerase. Viral regulatory genes encoding such regulatory proteins may act in trans as well as in cis, that is, these proteins may trans-activate genes residing on a physically separate molecule of nucleic acid.
A description of the role of the regulatory genes of human immunodeficiency virus 1 (HIV-1) serves to illustrate the sophistication of such regulatory mechanisms. A DNA copy of the viral genome is integrated into a chromosome of a resting T cell, and remains latent until a T cell mitogen or a cytokine induces synthesis of the cellular NF-kB family of DNA-binding proteins. NF-kB then binds to the enhancer present in the integrated provirus, thereby triggering transcription of the HIV-1 regulatory genes. One of these, tat, found in all lentiviruses, codes for a protein specific for a responsive element, TAR, within the provirus, greatly augmenting (trans-activating) the transcription of all viral genes (including tat itself). A positive feedback loop is thereby established that stimulates the production of HIV RNA transcripts. Moreover, by interacting with TAR present in all viral mRNAs as well as in the proviral DNA, tat also enhances translation. The HIV-1 regulatory protein Rev plays a different role to modulate gene expression by regulating the nuclear export of viral mRNAs. Although the control of lentivirus transcription may be unusually complex compared to many RNA viruses, these viruses contain only 9 genes, compared with up to 200 in the case of some DNA viruses.

Post-transcriptional Processing

Primary RNA transcripts from eukaryotic DNA are subject to a series of post-transcriptional alterations in the nucleus prior to export to the cytoplasm as mRNA. First, a cap, consisting of 7-methylguanosine (m$^\text{7}$Gppp), is added to the 5' terminus of the primary transcript; this cap structure facilitates the formation of a stable complex with the host 40S ribosomal subunit, necessary for the initiation of translation. Second, a sequence of 50 to 200 adenylate residues is added to the 3' terminus. This poly(A) tail acts as a recognition signal for processing and the transport of mRNA from the nucleus to the cytoplasm, and assists in the stabilization of mRNA against cytoplasmic degradation by ubiquitin. Third, a methyl group is added at the sixth position to about 1% of the adenylate residues throughout the RNA (methylation). Fourth, introns are removed from the primary transcript and the exons are linked together in a process known as splicing, an important mechanism for regulating gene expression in nuclear DNA viruses. A given RNA transcript can have two or more splicing sites and, moreover, be spliced in several alternative ways to produce a variety of mRNA species coding for distinct proteins; both the preferred poly(A) site and the splicing pattern may change in a regulated fashion as infection proceeds. For example, the HIV-1 protein Rev assists the nuclear export of unspliced or singly spliced (intron-containing) viral mRNA; thus, early in the replication cycle, the mRNA present in the cytoplasm is largely doubly spliced, while later in the cycle after Rev accumulates, a temporal switch in mRNA species occurs.

Special mention should be made of an extraordinary phenomenon known as cap-snatching. The transcriptase of influenza virus, which also carries endonuclease activity, steals the 5'-methylated caps from newly synthesized cellular RNA transcripts in the nucleus and uses these host cell RNA sequences as primers for initiating transcription from the viral genome.

The rate of degradation of mRNA provides another possible level of regulation. Not only do different mRNA species have different half-lives but the half-life of a given mRNA species may change as the replication cycle progresses.

Capped, polyadenylated, and processed monocistronic viral mRNAs bind to ribosomes and are translated into protein in the same fashion as cellular mRNAs. The sequence of events has been closely studied in reovirus-infected cells. Each monocistronic mRNA molecule binds via its capped 5'-terminus to the 40S ribosomal subunit and this then moves along the mRNA molecule until reaching the initiation codon. The 60S ribosomal subunit also binds, together with methionyl-transfer RNA and various initiation factors, after which translation proceeds.

Post-translational Modifications

Most viral proteins undergo various sorts of post-translational modification such as phosphorylation (for nucleic acid binding), fatty acid acylation (for membrane insertion), glycosylation, myristylation, or proteolytic cleavage. Newly synthesized viral proteins must also be transported to the various sites in the cell where they are needed, for example, into the nucleus in the case of viruses where the nucleus is the major site of replication. The sorting signals that direct intracellular trafficking are only now beginning to be understood, as are the polypeptide chain-binding proteins (molecular chaperones) that regulate folding, translocation, and assembly of oligomers of viral as well as cellular proteins.

Post-translational Cleavage

The polycistronic viral RNA is translated directly into a single polyprotein in the case of the positive-sense picornaviruses and flaviviruses. This large molecule carries protease activity that cleaves the polyprotein at defined recognition sites into smaller proteins. The first cleavage steps are carried out while the polyprotein is still associated with the ribosome. Some of the larger intermediates exist only fleetingly; others are functional for a short period but are subsequently cleaved by additional virus-coded proteases to smaller proteins with alternative functions. Post-translational cleavage occurs in several other RNA virus families, for example, togaviruses and calciviruses, in which polyproteins corresponding to large parts of the genome are cleaved. Some viruses encode several different proteases. Most are either trypsin-like (serine or cysteine
proteases), pepsin-like (aspartyl proteases), or papain-like (thiol proteases).

Cellular proteases, present in organelles such as the Golgi complex or transport vesicles, are also vital to the maturation and assembly of many viruses. For example, cleavage of the hemagglutinin glycoprotein of orthomyxoviruses or the fusion glycoprotein of paramyxoviruses is essential for virion infectivity.

**Glycosylation**

Viruses frequently exploit those cellular pathways normally used for the synthesis of host cell secretory glycoproteins. The amino terminus of viral envelope proteins contains a sequence of 15 to 30 hydrophobic amino acids, known as a signal sequence, which facilitates binding of the growing polypeptide chain to a receptor site on the cytoplasmic side of the rough endoplasmic reticulum and its passage through the lipid bilayer to the lumenal side. Oligosaccharides are then added in N-linkage to certain asparagine residues of the nascent polypeptide by en bloc transfer of a mannose-rich core of preformed oligosaccharides, and glucose residues are removed by glycosidases (called trimming).

The viral glycoprotein is then transported from the rough endoplasmic reticulum to the Golgi complex. Here the core carbohydrate is further modified by the removal of several mannose residues and the addition of further N-acetylglucosamine, galactose, and the terminal sugars, sialic acid, or fucose. The completed side-chains are a mixture of simple oligosaccharides (also called high mannose oligosaccharides) and complex oligosaccharides which are usually N-linked (to asparagine) or less commonly O-linked (to serine or threonine). A coated vesicle then transports the completed glycoprotein to the cellular membrane from which the particular virus buds.

The precise composition of the oligosaccharides is determined, not only by the amino acid sequence and tertiary structure of the proteins concerned, but more importantly by the particular cellular glycosyl transferases active in the type of cell in which the virus happens to be growing.

**Assembly and Release**

All non-enveloped viruses of vertebrates have an icosahedral structure. The structural proteins of simple icosahedral viruses associate spontaneously to form structural units (called capsomers when considered morphologically), which then self-assemble to form capsids into which viral nucleic acid is inserted, often accompanied by conformational changes to the nascent capsid structure. Completion of the virion may also involve proteolytic cleavage of one or more species of capsid protein.

The mechanism of packaging viral nucleic acid into a pre-assembled empty procapsid has been well elucidated for adenoviruses. A particular protein binds to a nucleotide sequence at one end of the viral DNA known as the packaging sequence; this enables the DNA to enter the procapsid bound to basic core proteins, after which some of the capsid proteins are cleaved to produce the mature virion.

Most non-enveloped viruses accumulate within the cytoplasm or the nucleus and are released only when the cell eventually lyses (Fig. 4.7).

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**FIGURE 4.7** Maturation of enveloped viruses. (A) Viruses with a matrix protein (and some viruses without a matrix protein) bud through a patch of the plasma membrane in which glycoprotein peplomers have accumulated over matrix protein molecules. (B) Most enveloped viruses that do not have a matrix protein bud into cytoplasmic vesicles (rough endoplasmic reticulum [RER] or Golgi), then pass through the cytoplasm in smooth-walled vesicles and are released by exocytosis. Reproduced from MacLachlan, N.J., Dubovi, E.J., 2011. Veterinary Virology, fourth ed. Academic Press, London (Fig. 2.12), with permission.
All mammalian viruses with helical nucleocapsids, as well as some with icosahedral nucleocapsids (e.g., herpesviruses, togaviruses, and retroviruses) mature by acquiring an envelope by budding through cellular membranes.

Enveloped viruses may bud from the plasma membrane, from internal cytoplasmic membranes, or from the nuclear membrane; viruses that acquire an envelope within the cell are then transported within vesicles to the cell surface. Budding usually occurs through patches of membrane that contain viral glycoprotein(s) inserted into the lipid bilayer of the membrane. This occurs by lateral displacement of cellular proteins from that patch of membrane. The cleaved single molecules of viral glycoprotein associate into oligomers to form typical rod-shaped or club-shaped peplomers with a hydrophilic domain projecting from the external surface of the membrane, a hydrophobic trans-membrane anchor domain, and a short hydrophilic cytoplasmic domain projecting slightly into the cytoplasm.

In the case of icosahedral viruses, for example 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, a matrix protein attaches to the cytoplasmic domain of the glycoprotein peplomer, and the nucleocapsid protein recognizes the matrix protein to initiate budding.

Release of each enveloped virion does not breach the integrity of the plasma membrane, hence many thousands of virus particles can be shed over a period of several hours or days without significant cell damage. Many but not all viruses that bud from the plasma membrane are only slowly cytopathic; and non-cytopathic may be associated with persistent infections.

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 separated by lateral cell–cell tight junctions. These surfaces are chemically and physiologically distinct. Viruses that are shed to the exterior (e.g., influenza viruses) tend to bud from the apical plasma membrane, whereas others (e.g., lentiviruses, such as human immunodeficiency virus) bud through the basolateral membrane.
Flaviviruses, coronaviruses, arteriviruses, and bunyaviruses mature by budding through either membranes of the Golgi complex or the rough endoplasmic reticulum; vesicles containing the virus then migrate to fuse at the plasma membrane thereby releasing the virions by exocytosis (Fig. 4.8). 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 cistermae of the endoplasmic reticulum.

**SATELLITE VIRUSES AND VIROIDs**

Satellite viruses are subviral particles that contain a DNA or RNA genome coding for a capsid protein, but that are absolutely dependent upon the presence of another virus for replication. The vast majority are found among plants but a few have an impact on medical virology and public health. Replication of the dependoviruses (family Paroviridae, genus Dependovirus—adeno-associated viruses, now used as vectors for delivering heterologous genes of interest, e.g., vectored vaccines) for example, is dependent upon co-infection with an adenovirus—the adeno-associated virus produces coat protein but not the enzymes necessary for genome replication. Satellite viruses share little or no nucleotide sequence homology with the helper virus, yet can sometimes interfere with the replication of the helper virus.

Viroids are small, rod-like single-stranded RNA molecules with a high degree of secondary structure, approximately 250 to 450 bases in size, all sharing a common structural feature of a conserved central genomic region essential for replication. The RNA forms a hammerhead structure with the enzymatic properties of a ribozyme, an autocatalytic, self-cleaving molecule. This ribozyme function is used to cleave multimeric RNA structures produced during the course of replication (Chapter 22: Hepadnaviruses and Hepatitis Delta). Most are plant pathogens, remarkable in not coding for any protein. First described by Theodore Diener, it has been suggested that viroids may represent crucial intermediate steps in the evolution of RNA-based life forms from inorganic precursors.

**Hepatitis Delta Virus**

Hepatitis delta virus (HDV) is a unique example of a defective virus that requires co-infection with hepatitis B virus to provide its outer HBsAg-containing envelope. The HDV genome is a branched or rod-like, single-stranded RNA molecule similar to plant viroids in conformation, but unlike other viroids it codes for a single protein, the delta antigen. This nuclear phosphoprotein exists in two forms generated by RNA editing: the shorter form (195 amino acids) is required for HDV genome replication whereas the larger form (214 amino acids) is necessary for assembly and release from infected liver cells.

The HDV genome is thought to be replicated by the host cell RNA polymerase II enzyme via a rolling circle mechanism to produce a consecutive series of concatemers of first, antisense RNA and then, RNA of genome sense. These are immediately cleaved by the ribozyme domain in the RNA genome to generate new viral genomes: this is the only known example of a mammalian virus utilizing a ribozyme for genome replication (see Fig. 22.9).

**GENERATION OF GENETIC DIVERSITY**

A sample of any given virus inevitably contains a population of closely related genome sequences ("quasi-species"), replicating simultaneously at different and varying rates according to selection pressures. The molecular mechanisms involved in generating diversity include nucleotide substitution, insertion, or deletion; sequence duplication or deletion; genetic recombination; and genetic reassortment for viruses with segmented genomes. The error rate in nucleotide copying is approximately 1 in 10⁸ nucleotides for DNA viruses, and 1 in 10⁶ nucleotides for RNA viruses. This quasi-species population is then constantly varying its composition according to the most recent selection applied, providing the raw material for antigenic drift and for adaptation of the virus population to new hosts.

The selection properties that are desirable in vivo include the ability to replicate to high levels, the ability to be transmitted from host to host, evasion of the immune response, and resistance to host antiviral mechanisms and/or antiviral therapy. Not all of these properties are as necessary for successful replication in cell culture as they are in vivo, and virus stocks prepared in vitro are likely to include a different set of variants from those produced in vivo, say in experimental animal tissues. Serial passaging of wild-type virus through cell culture or a foreign host species has been a long used and successful way to generate virus strains with lowered virulence for the original host, to be used as "modified live-virus" vaccines.

Many different viral genes may be involved in such adaptations; for example, mutations in viral polymerases may affect replicative ability or resistance to antiviral drugs, changes in antigenic epitopes can lead to immune escape, changes in receptor ligands that affect receptor preference can alter many aspects of pathogenesis, and so on. Examples are discussed in Chapter 15: Emerging Virus Diseases.

**QUANTITATIVE VIRUS ASSAYS**

Advances in our knowledge of virus replication, pathogenesis, diagnosis, vaccine production, and many other areas would not have been possible without the tools to quantitate how much virus there is in a given sample or.
preparation. With the greater use of antiviral drugs, most notably in blood-borne diseases such as HIV, hepatitis B virus, and hepatitis C virus, the stage of disease and effectiveness of treatment are now routinely assessed by quantifying the level of virus (viral load) in the blood.

There are two approaches to measuring the titer of virus, either biological or physical. Physical assays measure actual virus particles, and include electron microscopy, hemagglutination, and serological assays for the amount of viral antigens; biological assays measure some viral function, for example, infectivity, reverse transcriptase activity. Tests performed on the same sample with different techniques will in some cases give significantly different results, and thus it is important to understand the reason for these differences.

The difference between the amount of virus detected using a physical assay such as particle counting by electron microscopy and a biological assay, for example a plaque assay (Fig. 4.10), is often referred to as the particle to plaque-forming unit (pfu) ratio. In virtually all instances, the number of particles exceeds the number determined in a biological assay, often with ratios greater than 1000:1. This is due to a number of reasons; first, not all virions may be capable of replication due to intrinsic defects, for example, because only a partial or defective genome is present, or the particle is empty, faulty capsid assembly, or because of lethal mutations in the genome; second, virions may have undergone environmental inactivation; third, the choice of cells for virus isolation and growth may not mimic the optimum intracellular environment of the natural target organ; fourth, the interaction between a fully viable virus particle and a permissive cell may not always successfully initiate infection and an excess of particles may be necessary to achieve a statistical chance of success.

Perhaps no other procedure has contributed as much to virology as the development of the plaque assay. The test was originally developed a century ago by Felix d’Herelle in his initial studies of bacteriophages and was subsequently adapted to mammalian viruses in 1953 by Renato Dulbecco and Marguerite Vogt. The assay is elegantly simple: serial 10-fold dilutions of a virus sample are made in a cell culture medium. These diluted samples are then added to preformed monolayer cells and incubated under agar or a carboxymethyl cellulose layer to prevent the spread of secreted virus. After 24 to 48 hours (or longer, according to the virus of interest), plaques of necrotic cells become visible under the light microscope. At an appropriate time, fixation and staining, for example, with crystal violet, reveals clearly visible holes in the monolayer, each corresponding to a focus of necrotic cells initiated by one viable virus particle. Serial dilution of the virus preparation facilitates the counting of discrete plaques so that, knowing the dilution, volume tested, and the plaque size, the concentration (titer) in the original sample can be determined. Immunohistochemical staining procedures using specific antisera can be used as an alternative for visualizing plaques formed by non-lytropic viruses.

With the development of real-time PCR assays, the concentration of viral nucleic acid in a test sample can now be measured in virtually any context. By comparison with copy number controls, 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, importantly, it does not necessarily relate to the infectivity of the sample.

Chapter 10: Laboratory Diagnosis of Virus Diseases, describes a further range of assays that are used in a diagnostic context, most of which can be also used both quantitatively and for research applications.

DEFECTIVE INTERFERING VIRUSES

This chapter has hitherto described how, during virus replication, non-infectious particles that lack the full genetic information essential for infectivity, are commonly produced—analogous to the assembly of defective motor
vehicles from a car assembly plant. One special example involves defective interfering (DI) particles; these (1) lack the complete genome of the wild-type virus (deletion mutants), but (2) can still replicate in a new cell if the cell is also co-infected by wild-type virus which provides the missing function (by a process known as complementation). Such defective particles may then also (3) interfere with the ongoing replication of the wild-type helper virus, possibly due to possessing a competitive replication advantage over wild-type virus.

The production of DI particles is encouraged by infection at high multiplicity, where the chances of co-infection with full-length and defective viruses occurring in the same cell are increased. In fact, this phenomenon was first observed by Preben and Herdis von Magnus in 1944 with influenza virus; they found that very high multiplicities led to poor virus replication and the production of “interfering” virus stocks. A cyclical pattern may be observed, where DI particles increase in number as long as the culture has sufficient wild-type helper virus replication; however, as DI particles then inhibit the numbers of wild-type virus, the whole virus population diminishes, fresh rounds of wild-type virus replication can then occur and so a milieu for a return to DI replication is built up again. Despite several suggestions, it is not clear to what extent DI particles play a role in the pathogenesis of an ongoing infection in a host, for example, in modulating recovery from an acute infection or promoting chronic infection.

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