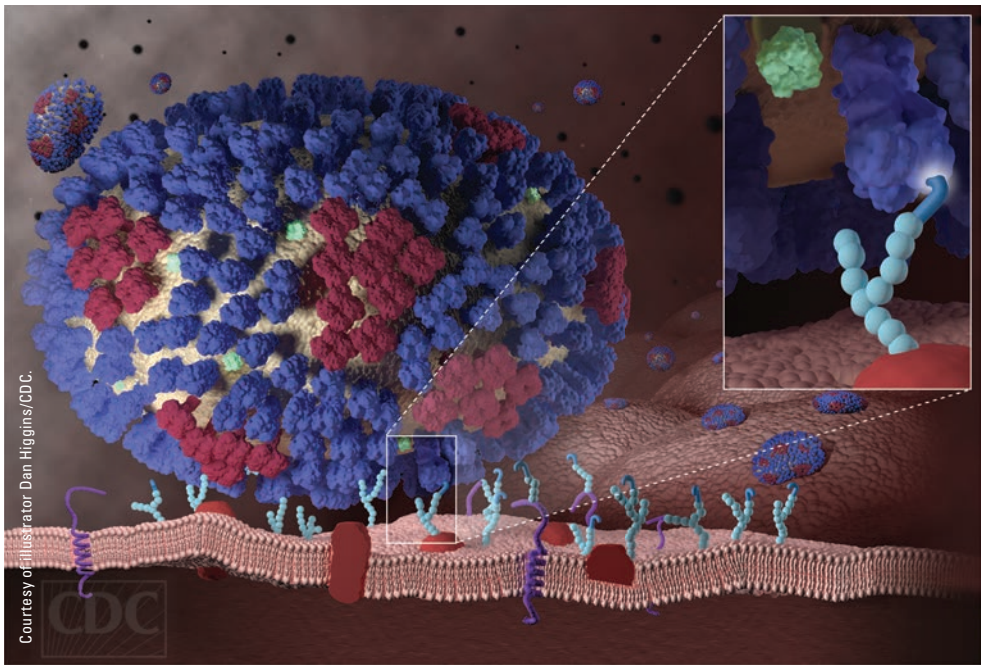
CHAPTER 3

Eucaryotic Molecular Biology, Cellular Hurdles, and How Viruses Hijack Host Cells

"A relatively small number of investigators have been preoccupied with the biology of viruses … and how they tick; these scientists are more sensitive to the … evolution of their symbiotic relations to their hosts."

—Joshua Lederberg, American molecular biologist (1925–2008)



Three-dimensional image of influenza A virus hemagglutinin (HA) surface protein attaching to sialic acid receptors present on the surface of a human cell within the nasal passages of the throat.

OUTLINE

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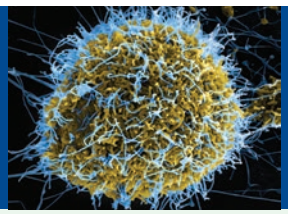
LEARNING OBJECTIVES

1. Describe the basic structure of a virus.
2. Summarize the molecular challenges that viruses must overcome in order to replicate within their hosts.
3. Explain why all viruses use the protein synthesis machinery of their host.
4. Summarize the general procedure for one-step growth curves.
5. List the key steps of the viral replication cycle, and define what happens at each step.
6. Explain why RNA viruses usually have higher mutation rates than DNA viruses.
7. Summarize why it is difficult to develop antivirals that have no toxic side effects.
8. Explain how viruses have been our "eyes" into cells.



CASE STUDY 1: THE MOTIVES OF EBOLA VIRUS

Undergraduates Kathy Gallo and Jaime Hernandez had enrolled in a BIO 315 (Virology) course because they were interested in Ebola virus after the unprecedented 2014 Ebola epidemic in West Africa. It was impossible for them to avoid paying attention to media hype about the epidemic. They had fears that hospital workers in the United States were not prepared to care for Ebola patients. After two nurses in Texas became infected while treating Ebola patient Thomas Eric Duncan from Liberia, they had even more concerns.

Kathy and Jaime were curious about how an infection with Ebola virus could cause death so quickly. They wanted to understand more about the pathogenesis of Ebola virus infections and began to do some research before the class started so that when the class began the professor for the course might be able to answer their questions.

The students quickly learned that Ebola virus infects and damages a variety of cells, such as the mucosal epithelial cells of the mouth, nose, and eyes and immune cells, such as monocytes, dendritic cells, and macrophages. The viruses are disseminated through blood and the lymphatic system. As they tried to understand why patients experienced uncontrolled bleeding, they discovered that Ebola viruses infect the endothelial cells lining blood vessels, resulting in blood loss. Infection by Ebola virus in tissues such as the liver and adrenal glands impacts the production of **clotting factors** and **steroids**. This affects blood pressure, ultimately causing vascular instability and shock.

Jaime and Kathy didn't think that viruses "intend" to cause disease, but they knew that viruses have motives to program host cells to replicate more of themselves. In

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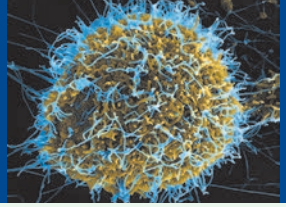


CASE STUDY 1: THE MOTIVES OF EBOLA VIRUS (continued)

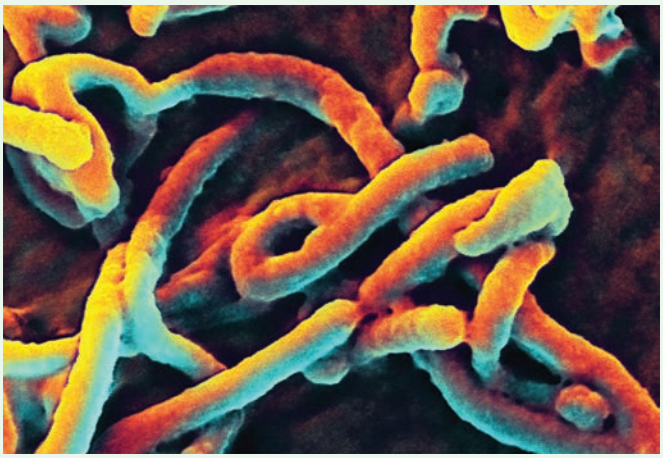
doing so, viruses steal the resources of their cellular hosts and manipulate or inhibit host cell processes in order to replicate. On their own, they spent time learning about the virion structure and genome of Ebola virus. Jaime was fascinated that it is an enveloped, helical-shaped virus about 80 nanometers (nm) in diameter and 800–1,000 nm in length, giving it a distinctive *spaghetti shape* when visualized by transmission electron microscope. This image is portrayed on the cover of this edition of *Understanding Viruses* (**FIGURE 1A**).

The genome of Ebola virus is a **negative, linear, single-stranded RNA (–ssRNA)** that is about 19 kilobases (kb) in length, consisting of seven genes that code for eight proteins. The structural proteins that make up the Ebola virus particle include two matrix proteins, **VP24** and **VP40**, a nucleoprotein (NP) that protects the genome, and two forms of glycoprotein (GP). Ebola virus also contains an RNA replication complex composed of **NP**, **VP30**, **VP35**, and **polymerase** (**L**). The genome and structure of an Ebola virus particle are shown in **FIGURES 1B** and **1C**, respectively.

Kathy spent a lot of time researching the replication cycle of Ebola virus. She knew that the damage to the host cell was the result of Ebola virus interactions with the host and immune system. Jaime was very interested in the replication cycle of Ebola virus because he knew that there were no antiviral drugs available to treat **Ebola virus disease (EVD)**. A few *experimental* drugs were approved for emergency use by the **World Health Organization** (**WHO**) to treat a small fraction of EVD patients. Kathy and Jaime discerned that therapeutic agents could be developed to target different steps of the replication cycle of Ebola virus.

The first step in a virus replication cycle is attachment. Because Ebola virus can enter more than one cell type, Jaime knew that Ebola virus entry would involve one or more host cell **receptors**.

After completing this chapter, you should be able to find resources to help answer Kathy and Jaime's questions as to how Ebola virus hijacks host cells in order to replicate. In doing so, you will be able to identify potential drug therapy targets for the treatment of EVD.



Courtesy of the National Institute of Allergy and Infectious Diseases.

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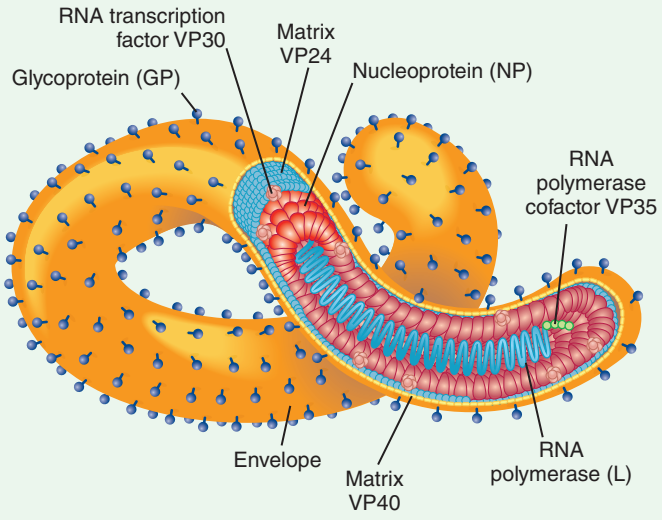

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FIGURE 1 **(a)** Digitally colorized scanning electron micrograph of Ebola virus budding from Vero cells. **(b)** Structure of the Ebola virus. It is an enveloped, long, slender, helical-shaped virus that contains a single linear –ssRNA genome. Anchored within the lipid bilayer membrane is the glycoprotein (GP) that forms spikes on the surface of the virion. GP is used for attachment to host proteins. Other structural proteins that make up the Ebola virus particle are two matrix proteins in the space between the core and the envelope, VP24 and VP40, and a nucleoprotein (NP) that protects the genome. The RNA replication complex is packaged within the particle. It is composed of NP, VP30, VP35, and viral RNA polymerase (L). **(c)** The genome of Ebola virus is –ssRNA, linear, and approximately 19 kilobases in length. It contains seven genes that code for eight proteins: two forms of GP, the secreted or sGP that is not part of the virion and the nonsecreted GP (which are the spikes protruding from the virion); NP, matrix proteins VP24 and VP40 and RNA replication complex that consists of VP35 (RNA polymerase cofactor), VP 30 (transcription factor), and the viral RNA polymerase (L).

See Case Study 1 Questions at the end of the chapter.

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Viruses are inert (inactive) outside of their host cells. They can attach and enter (infect) all types of cells, including animal, plant, insect, and bacterial cells. This text mainly focuses on human viruses, but it also addresses some animal, plant, fish, bird, insect, and bacterial viruses as well. Before studying individual viruses in depth, though, we must first analyze the basic structure of a human or mammalian virus. Viruses that infect humans or other mammals typically share a few common features:

- Small in size (nanometer range)
- Able to pass through ultrafilters that trap most known bacteria
- Completely dependent upon the host cell for replication
- Usually contain one type of nucleic acid (RNA or DNA)
- Possess receptor-binding proteins present on the surface of the virus particle

Virus particles consist of a nucleic acid genome protected by a protein shell that gives the virus particle its strong structure. The shell, referred to as the **capsid**, can contain other proteins, such as receptor or viral attachment proteins, that allow the virus to adhere to the outside of a host cell. Human or mammalian virus anatomy is different from that of bacteriophages.

The capsid protects the nucleic acid genome of the virus from a harsh environment that is laden with ***nucleases***. Any unprotected nucleic acids present in the environment are destroyed or inactivated by nucleases. The capsid and associated genome constitute the **nucleocapsid**. Many viruses also code for and carry their own polymerases to replicate the viral genome. **DNA polymerases**, **RNA polymerases**, or **reverse transcriptases** catalyze the formation of polynucleotides of DNA or RNA using an existing strand of DNA or RNA as a template. This is necessary because the host may not contain a polymerase that will replicate the viral genome (host cell constraints are discussed in more detail later in the chapter).

Viruses that do not contain an envelope are **naked viruses** (**FIGURE 3-1A**). Examples of naked viruses that infect humans are noroviruses and adenoviruses. **Noroviruses** cause **gastroenteritis** (stomach pain, nausea, diarrhea, and vomiting), "stomach flu," or **winter vomiting disease**, and adenoviruses cause mild respiratory illness. **Adenoviruses** can cause cold-like symptoms, sore throat, bronchitis, pneumonia, diarrhea, and pink eye (**conjunctivitis**). Naked viruses are environmentally stable. Noroviruses can remain infectious on a person's hands for 2 hours and persist on surfaces for up to 2 weeks. The **infectious dose**, the number of infectious particles it takes to cause an infection and cause illness, for noroviruses is low (≤ 10). The highest risk of exposure occurs during outbreaks in schools, day cares, retirement homes, hospitals/healthcare facilities, and restaurants and on cruise ships.

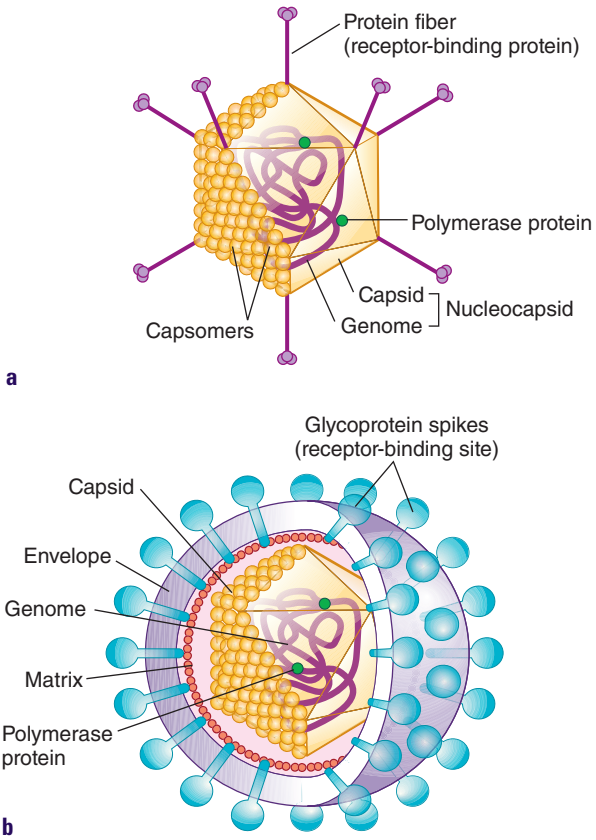

FIGURE 3-1 **(a)** Structure of a naked (nonenveloped) virus that consists of a nucleocapsid that may contain protein fibers that are involved in attachment to host cell receptors. **(b)** Structure of an enveloped virus that contains an envelope bridged by matrix proteins to the nucleocapsid. Many virus particles contain polymerases bound to the viral genome for its replication.

Enveloped viruses contain a lipid bilayer membrane wrapped around the nucleocapsid of the virus particle. Matrix proteins, found within many enveloped viruses, add rigidity to the virus particle and act as a bridge between the nucleocapsid and the viral proteins embedded in the envelope (**FIGURE 3-1B**). *In doing so, matrix proteins secure the internal nucleocapsid to the envelope, which may be critical for budding and release of viruses from host cells*. The entire structure of the virus—the genome, capsid, and (where present) the envelope—constitute the **virion**, or the completely assembled, *infectious* virus particle. The lipid bilayer membrane is stolen from the host cell as newly assembled viruses bud and exit from the host cell following infection and replication. This lipid bilayer may come from the host cell's plasma (outer) membrane, the nuclear membrane, or its trans-Golgi network.

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3.1 Genes Required for Assembly of Infectious Virus Particles

Viral genomes are small and diverse compared to those of their hosts. Viruses have evolved ways to replicate that are economical. For example, a virus may require only one type of capsid protein. The protein is used over and over again to build the capsid or protein shell of the virus. Therefore, this task can be accomplished with only one gene coding for the capsid protein. In addition to a gene that codes for the capsid protein, genes are needed to code for the viral receptor-binding protein used for attachment to the host cell, and many viral genomes contain a polymerase gene that is required for viral genome replication. This means that, in theory, a viral genome might contain as few as three genes, but most viral genomes code for more than three gene products.

The genome length of viruses varies. The size of viral genomes composed of **single-stranded RNA (ssRNA)** ranges from 10,000 to 27,000 bases (or 10–27 kb) in length (e.g., the West Nile virus +ssRNA genome is ~10.2 kb in length). These viral genomes generally contain fewer genes (usually less than a dozen). It has been hypothesized that genomes composed of single-stranded nucleic acids are more fragile than those composed of double-stranded nucleic acids. In general, this is true for viruses containing ssRNA and ssDNA genomes. Viral genomes consisting of **double-stranded DNA (dsDNA)** are classified as either small or large. For example, polyomavirus dsDNA genomes are a mere 5 kb in length, coding for 6 gene products. Poxvirus dsDNA genomes are large, consisting of about 200 kb pairs, coding for approximately 200 gene products. An exception is the genomes of **giruses** like Mimivirus, which are enormous. The Mimivirus genome is 1.2 megabase (Mb) pairs in length, believed to code for about 911 functional proteins.

Why Are Viruses Dependent on a Host for Replication?

Viral genomes may contain as few as a handful of genes or as many as 200. What cellular functions or processes to accomplish the task of generating new viruses do viruses need that would take more than this number of genes? To answer this question, a review of the central dogma of molecular biology, eucaryotic DNA **replication**, RNA **transcription**, and **translation** (protein synthesis) is necessary. Think about how many gene products or proteins are necessary to carry out each of these processes.

3.2 Molecular Biology Review

To replicate or produce more virions, viruses infect and hijack a host cell to direct its machinery for help. Some viruses use the host's replication and transcription machinery, whereas others contain genes that code for different types of **polymerases** that are used to replicate viral genomes and transcribe **messenger RNAs** (**mRNAs**) that will be translated by the cellular protein synthesis machinery (e.g., ribosomes, **transfer RNAs** [**tRNAs**]). *All viruses are dependent on their host for the translation machinery and energy supplies*.

Cellular Processes Are Localized

It is important to remember that the processes of replication, transcription, and translation are *localized* in eucaryotic host cells (**FIGURE 3-2A**). DNA replication and RNA transcription occur within the **nucleus** of the host cell. Processed (spliced) mRNAs exit the nucleus and are translated in the **cytoplasm** of host cells by the ribosomal machinery. A virus in need of the cellular machinery to replicate its genome or transcribe viral mRNAs must ensure that its viral nucleic acid is in the nucleus of the host cell. The viral genome is replicated and the viral mRNAs are transcribed and processed in the nucleus. Subsequently, the processed viral mRNAs exit the nucleus through the nuclear pores into the cytoplasm where they are translated into viral proteins by the cellular translational or ribosomal machinery. *Let's briefly review the central dogma of molecular biology and the processes of eucaryotic DNA replication, RNA transcription, and translation*.

5′→ 3′ Directionality of Nucleic Acid Synthesis

All nucleic acids have two distinct ends: the 5′ (5-prime) and 3′ (3-prime) ends, which refers to the 5′ end that bears a phosphate group and the 3′ end, a hydroxyl group. The orientation of all nucleic acid synthesis *in vivo*, for both RNA and DNA, is in a 5′ → 3′ direction. During nucleic acid synthesis, a phosphodiester bond forms between the 3′ carbon of one nucleotide and the 5′ carbon of another nucleotide, resulting in the formation of the "sugar-phosphate backbone." DNA or RNA polymerases add nucleotides to the 3′ end of the previously incorporated nucleotide base (**FIGURE 3-2B**).

The Central Dogma of Molecular Biology

> *"The central dogma, enunciated by Crick in 1958 and the keystone of molecular biology ever since, is likely to prove a considerable oversimplification."*
>
> —Unknown author of "Central Dogma Reversed," *Nature*, June 27, 1970

All host genomes are composed of dsDNA, in contrast to viral genomes, which may be dsDNA, ssDNA, **double-stranded RNA** (**dsRNA**), or **single-stranded RNA** (**ssRNA**). The human genome is composed of 23 pairs of **chromosomes**, totaling about 3 billion DNA base pairs. A paradigm known as the **central dogma of molecular biology** is that DNA is first transcribed into mRNA by RNA polymerase II and then mRNA is translated into protein by the cellular translational

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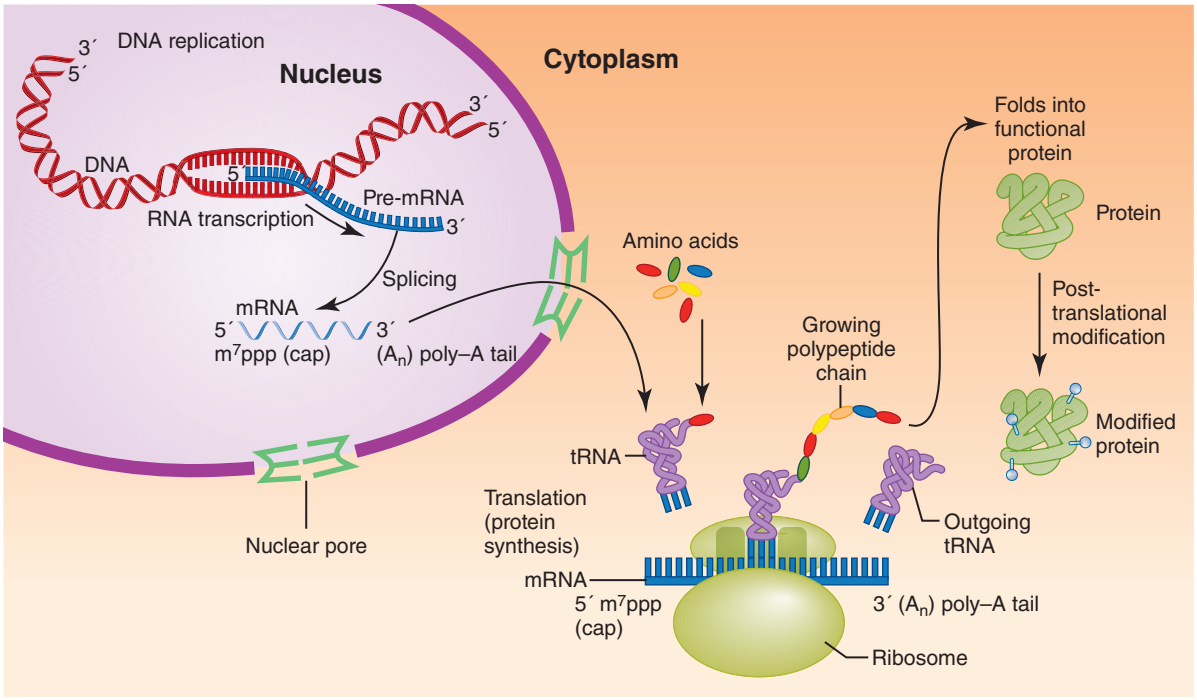

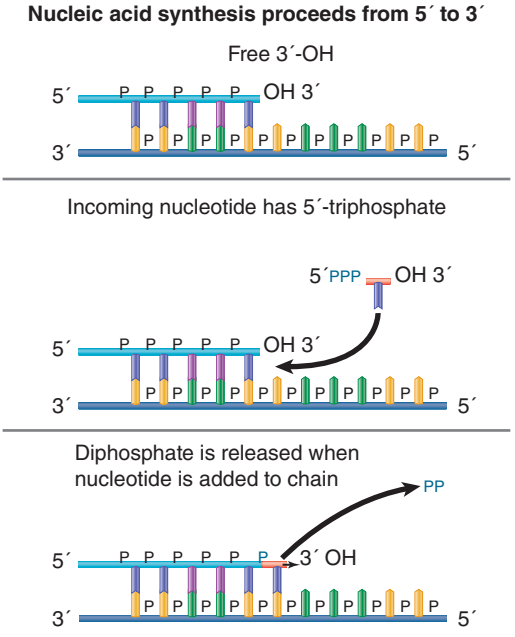

FIGURE 3-2 **(a)** Diagram showing that DNA replication and RNA transcription occur in the nucleus and translation takes place in the cytoplasm of a eucaryotic cell. Messenger RNA (mRNA) exits the nucleus through the nuclear pores, where it is translated in the cytoplasm by ribosomes. Transfer RNA (tRNA) carries the next amino acid to be attached to a growing polypeptide chain to the ribosome that recognizes and matches a three-nucleotide sequence in the mRNA (codon) and a complementary sequence in the tRNA (the anticodon) and transfers the growing polypeptide chain to the incoming amino acid while it is still attached to the tRNA. The finished polypeptide is folded into a functional three-dimensional shape. Some proteins undergo posttranslational modification. **(b)** All nucleic acid synthesis *in vivo* occurs in the 5′ → 3′ direction.

machinery (Figure 3-2). Although this central dogma is generally true, exceptions can be found.

The DNA sequencing of the human genome revealed that a mere 1.5% of the genome consists of 20,000–25,000 genes, or protein-coding sequences. A **gene** is a sequence within the genome that gives rise to a discrete, functional protein. The vast majority of the remaining genomic DNA is transcribed into noncoding RNAs that *was* described as "junk" or **introns** (noncoding intervening sequences within coding genes that also became known as "functionless gene copies," or **pseudogenes**) in the genome. The past few decades of research have brought forth an explosion of information that has changed researchers' views on the significance of this noncoding RNA. *The noncoding RNAs have been linked to various human diseases, including cancers, and to genome instability during aging.*

The non-protein-coding DNA sequences of the human genome consist of other DNA elements, such as introns (about 26%); **promoters** and **enhancers** that regulate gene expression; structural DNA sequences such as **telomeres** present on the ends of chromosomes; noncoding RNAs such as **ribosomal RNA (rRNA)** and tRNAs used

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in translation; **micro-RNAs** (**miRNAs**) used in gene silencing; and **small nuclear RNAs** (**snRNAs**) used in splicing of introns from primary genomic transcripts, or **pre-mRNAs**. Nearly half (45%) of the human genome consists of (mostly defunct) **transposable genetic elements** that are ~100–10,000 base pairs in length that move around the genome by a **cut-and-paste** (**transposition**) **mechanism** (**FIGURE 3-3A**). Eucaryotes have two types of transposable elements that are classified based on their mechanism of transposition: class I **retrotransposons** and class II **DNA transposons**.

The class I retrotranposon elements encode a reverse transcriptase and are transposed through an RNA intermediate. Reverse transcriptases are enzymes that synthesize a DNA molecule from the code supplied by an RNA molecule. The class II DNA transposons represent 2.8% of the human genome. The DNA transposons encode for a **transposase** that performs a cut-and-paste mechanism to insert itself into specific sites of the DNA genome.

The class I transposable elements can be further dissected into **LTR retroviral-like retrotransposons** (8.3% of the human genome), which behave similar to retroviruses, and **non-LTR nonretroviral retrotransposons** (33.7% of the human genome; **FIGURE 3.3B**). The presence of LTR retroviral-like retrotransposons is commonly believed to have originated as a result of an ancient retroviral infection in the germ line. In the human genome, the LTR retroviral-like retrotransposons are also called **human endogenous retroviruses (HERVs)**. The reverse, or "retro," flow of genetic information from RNA to DNA is a hallmark of the retrovirus replication cycle, which will be discussed in detail in Section 3.5. The discovery of retroviruses, which reverse-transcribe RNA into DNA using virally encoded reverse transcriptases, resulted in an exception to the central dogma. *Since the discovery of reverse transcriptases of retroviruses, other reverse transcriptases have been discovered in host cells, such as the* ***human telomerase reverse transcriptase*** *(**TERT**), which maintains the ends of telomeres.*

Eucaryotic DNA Replication

Eucaryotic cells have more than a dozen DNA polymerases that synthesize the dsDNA. Two of these—DNA polymerases α and δ—are important for the replication of eucaryotic chromosomes. Chromosomal dsDNA is copied/read 3′ → 5′ and synthesized in a 5′ → 3′ direction by DNA polymerases inside of the nucleus of the cell. DNA polymerases are sometimes referred to as **DNA-dependent DNA polymerases** because they synthesize DNA from a DNA template. The following are additional properties of cellular DNA polymerases:

- They cannot "initiate *de novo* DNA synthesis" (they require a RNA primer to do so).
- They have high fidelity of copying (generating one error in every 10^9 base pair replications).
- They may possess proofreading ability (3′ → 5′ **exonuclease** [editing] activity to remove incorrect nucleotides, which are subsequently replaced by the correct nucleotides).
- They may be capable of **helicase** (unwinding) and **primase** (synthesis) activities.
- They are localized and active in the nucleus of cells.

The process of DNA replication in eucaryotes requires additional cellular proteins such as primases (to synthesize RNA primers), ssDNA-binding proteins

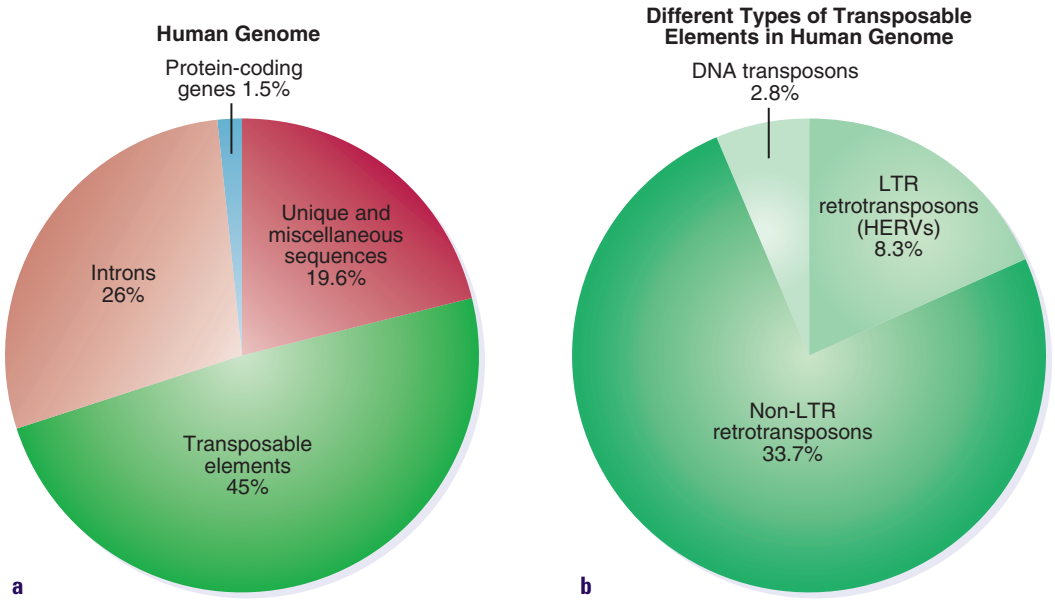

FIGURE 3-3 **(a)** Pie chart showing the composition of the main components of the human genome. **(b)** Pie chart showing the types and percentage of each type of transposable element present in the human genome.

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(to protect the dsDNA from nucleases during replication), and ligases (to join together **Okazaki fragments**/lagging strands).

Eucaryotic RNA Transcription and RNA Splicing

A gene does not directly generate a protein. Remember from the central dogma of molecular biology that a gene (DNA) codes for RNA, which may in turn code for a protein. Eucaryotic cells convert the information in DNA into RNA by the process of transcription. Enzymes called *DNA-dependent RNA polymerases* catalyze the RNA synthesis. Eucaryotic cells contain three different types of DNA-dependent RNA polymerases: RNA polymerases I, II, and III. **RNA polymerase I** synthesizes rRNA. **RNA polymerase II** synthesizes pre-messenger RNA (pre-mRNA) and some small nuclear RNAs (snRNAs). **RNA polymerase III** synthesizes transfer RNA (tRNA), 5S rRNA, and other small RNAs. We will focus on RNA polymerase II because it is responsible for synthesizing cellular mRNA that is translated by ribosomes. *Viral mRNAs must be biochemically similar to cellular mRNAs so that the ribosomes of the cell can recognize viral mRNAs and translate them using the host protein synthesis machinery (e.g., ribosomes, tRNAs). Structurally, viral mRNAs can be very different, from their 5′ and 3′ end structures that are synthesized by viral RNA-dependent RNA polymerases to the complex secondary structures that direct ribosome binding.*

RNA polymerase II of eucaryotic cells transcribes mRNA from DNA in the nucleus. RNA polymerase II does not bind directly to eucaryotic DNA. The DNA contains upstream and downstream sequences (elements) prior to the start of transcription that **transcription factors** recognize and bind to, forming a **preinitiation complex** that recruits the binding of RNA polymerase II to the **core promoter** region of a specific gene. A typical eucaryotic gene with its upstream sequences and core promoter is shown in **FIGURE 3-4**. The AT-rich **TATA box**, a common component of the core promoter, is located about 25 base pairs upstream of the starting point/initiation of transcription. Enhancers are DNA elements located outside of the promoter that stimulate the frequency of transcription of genes by RNA polymerase II. Enhancers differ from promoters in that their sequences are orientation and position independent. Enhancers are located upstream (before) or downstream (after) the promoter of the gene being transcribed.

The characteristics of RNA polymerase II are that it

- Binds the transcription preinitiation complex in the promoter region of DNA.
- Can **initiate** the *de novo* synthesis of RNA (i.e., it does not require a primer).
- Copies/reads 3′→ 5′ and synthesizes RNA 5′→3′.
- Synthesizes and processes mRNA in the nucleus of the cell.
- Is error prone (1 mistake in 10,000 bases).
- Has no proofreading ability.
- Is recruited by transcription factors to the DNA promoter.

The host cell's RNA polymerase II synthesizes a large primary (or precursor) **pre-mRNA transcript** (Figure 3-4). As the pre-mRNA is being transcribed (after ~30 nucleotides), the 5′ end of the pre-mRNA is modified, or "capped," by the addition of a methylated guanine nucleotide (5′m^{7}Gppp). The cap protects the mRNA from degradation and is recognized by cellular ribosomes during translation. The 3′ end of the pre-mRNA transcript is cleaved about 20–30 nucleotides past the polyadenylation sequence, and then a poly(A) polymerase adds 100–200 residues of adenylic acid to the 3′ end of the mRNA called a **poly(A) tail** (**FIGURE 3-5**). The poly-A tail may also protect the mRNA from degradation. The poly(A) tail usually becomes shorter as an mRNA ages in the cytoplasm; when it reaches a minimal length, the mRNA will be degraded by cellular nucleases. Certain viruses, such as vaccinia virus, spend their entire replication cycle in the cytoplasm of the host cell. Vaccinia encodes all of the components of its transcriptional machinery to generate capped and polyadenylated viral mRNAs.

Genes consist of **exons** and introns. Exons are the sequences representing the mature mRNA. Introns are intervening sequences that are removed from the pre-mRNA, resulting in the mature mRNA. **Splicing** occurs when introns that are excised (removed) from the

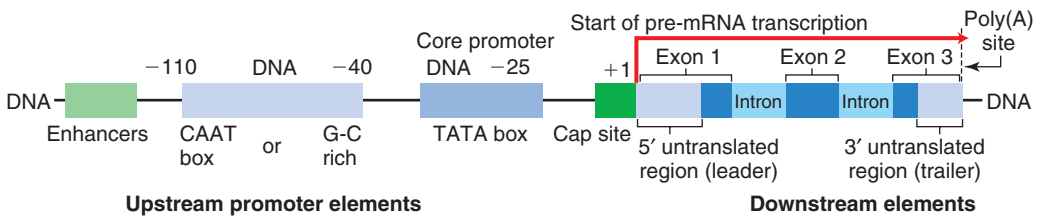

FIGURE 3-4 A typical eucaryotic gene containing an upstream enhancer, an upstream promoter, and core promoter elements along with the transcribed region of the gene. Transcription factors bind to the upstream promoter elements and serve to bind RNA polymerase II and to position it correctly to the starting point. Promoters may be stimulated by enhancers that can act at great distances and in either orientation of a gene. A large primary pre-mRNA is transcribed that will be processed before it is a mature mRNA that is exported into the cytoplasm for translation.

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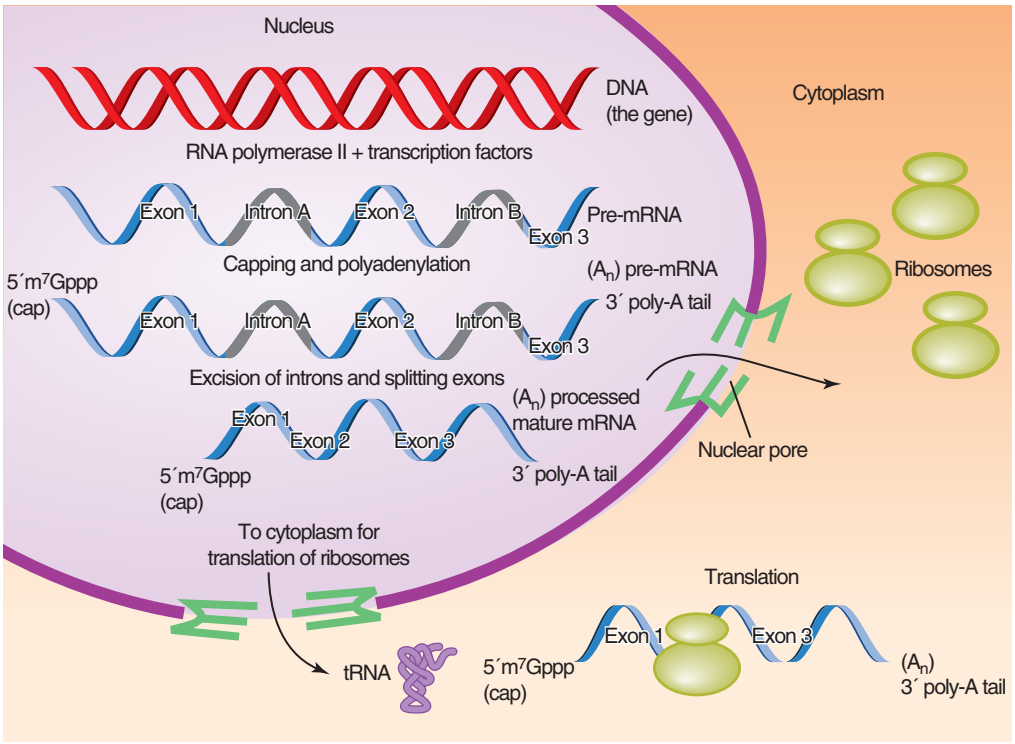

FIGURE 3-5 Eucaryotic transcription occurs in the nucleus. After the mRNAs are processed, the mature (capped, polyadenylated, and spliced) mRNA exits the nuclear pores and enters the cytoplasm of the cell to be translated by the translational machinery.

modified pre-mRNA and the exons are connected into a continuous mRNA. Splicing reactions require snRNAs, proteins, and adenosine triphosphate (ATP). After this event, the completed mRNAs are exported from the nucleus into the cytoplasm of the cell where they will be translated into proteins. Only about 5% of the originally transcribed mRNA exits the nucleus (Figure 3-5). The discovery of intron removal by splicing was made independently by two different teams of researchers studying the viral mRNAs produced in **HeLa cells** (a tissue culture human cell line) infected with adenovirus 2 (**VIRUS FILE 3-1**).

Eucaryotic Translation

Translation, or protein synthesis, is the decoding of mRNA into protein. Translation takes place in the cytoplasm of cells and involves all three types of RNA: transfer RNA (tRNA), ribosomal RNA (rRNA), and messenger RNA (mRNA). It is a three-step process:

1. **Initiation**: Formation of the initiation complex.
2. **Elongation**: Synthesis of the polypeptide/protein.
3. **Termination**: The mRNA contains an in-frame stop codon, and the completed polypeptide/protein is released.

The focus of this review will be on initiation of translation because *all viruses are dependent upon the host cell translation machinery to produce viral proteins*. Initiation is the key event in translation because viruses must hijack the host translational machinery to produce their own proteins. Viruses do not have the genetic capacity to encode the proteins necessary for eucaryotic protein synthesis. In addition to ribosomes, rRNA, tRNA, and mRNA, there are at least a dozen **eucaryotic translation initiation factors** (**eIFs**) involved in the initiation of translation in eucaryotes (**TABLE 3-1**). The eIFs catalyze individual steps in the cap-dependent pathway (reviewed later). *Viruses evolved to utilize many different strategies to ensure that their mRNA is preferentially translated over the myriad cellular mRNAs.*

Translation and Open Reading Frames

Regions of DNA are transcribed into mRNA, and continuous open reading frames (ORFs) are created when introns are removed from the DNA (**FIGURE 3-6A**). The genetic code is read in nonoverlapping triplets. The reading frame that is used in translating an mRNA (in eucaryotes) is often the longest ORF. The ORF consists of triplets representing amino acids. An mRNA that is translated into protein has an ORF that starts with a special initiation codon (AUG) and continues through a series of triplets representing amino acids until it ends at one of three types of termination codons (UAA, UGA, UAG) (**FIGURE 3-6B**). An ORF that cannot be read into protein because a termination codon occurs is blocked.

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VIRUS FILE 3-1 RNA Splicing: A Teachable Moment by Adenovirus 2

The 1977 discovery that DNA encodes mRNAs which are interrupted by ***introns*** *(intervening sequences) that are removed from pre-mRNA transcripts was one of the most unexpected discoveries in the history of molecular biology*. Prior to this time, molecular cloning did not exist, and scientists knew that transcribed mRNAs found inside the nucleus of eucaryotic cells were longer than mRNAs found in the cytoplasm of cells. They believed that the mRNAs were cleaved in the nucleus before they were exported to the cytoplasm.

At first, it was assumed that the sequences from the 5′ or 3′ end of the mRNAs were trimmed, resulting in the shortened mRNAs. This was ruled out after experiments showed that both the nuclear pre-mRNA and the mRNAs in the cytoplasm contained 5′m^7G, or both. This meant that if both ends of the pre-mRNA molecule were conserved, then one or more segments within the pre-mRNA were removed and the remaining segments would be rejoined to form the shortened mRNA. It raised the possibility that a splicing mechanism could be used to explain this type of pre-mRNA processing. It was an unprecedented line of thought, and researchers were reluctant to accept this explanation until two independent teams of researchers were able to show convincing visual evidence that splicing does indeed occur.

The first experiments to demonstrate splicing were done by Berget, Moore, and Sharp (Massachusetts Institute of Technology [MIT], Cambridge, Massachusetts) and Chow, Gelinas, Broker, and Roberts (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). Both teams of investigators infected HeLa cells with adenovirus 2. Adenovirus 2 is a naked virus that contains a dsDNA genome with 35,000 base pairs. Studying adenoviruses was much simpler than studying the processing of cellular mRNAs transcribed from a eucaryotic genome.

The dsDNA genome of adenovirus 2 can be directly isolated from adenovirus 2 particles. Adenovirus 2 has a replication cycle that can be divided into early and late phases. The cellular RNA polymerase II transcribes the viral mRNAs, and cellular enzymes add the 5′m^7G cap and 3′ poly(A) tail. During the late phase of infection, HeLa cells infected with adenovirus 2 produced large amounts of viral mRNAs that code for capsid proteins such as **hexon proteins** that can be purified from polysomes (**FIGURE 1**) and separated from other adenovirus 2 mRNAs. The purified hexon mRNAs were then incubated with adenovirus 2 DNA that had been cleaved into dsDNA fragments with **restriction enzymes** and denatured into ssDNA. Adenovirus 2 hexon mRNA–DNA hybrids formed, mapping the hexon mRNAs to the location of their complementary sequences on the viral DNA genome (**FIGURE 2**). The hybrids formed were visualized using transmission electron microscopy (TEM). This technique is called **R-loop mapping**.

To the researchers' surprise, the 5′ end of the hexon mRNAs failed to anneal or map to form a single continuous stretch of mRNA–DNA hybrids. Instead, three long loops of unhybridized DNA formed, skipping intervening sequences on the DNA (**FIGURE 3A**. electron micrograph **B**. schematic of electron micrograph). Each loop corresponded to a region in which there was no complementary viral mRNA sequences, which suggested that the

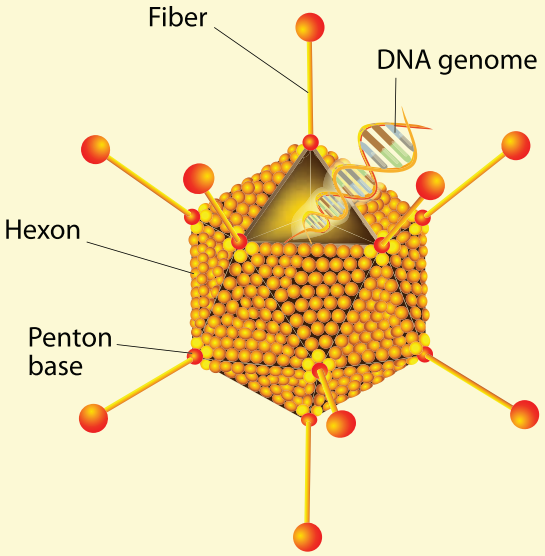

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FIGURE 1 Structure of adenovirus 2 showing hexon protein, which is one type of structural protein that makes up the capsid of the virus particle.

(*continues*)

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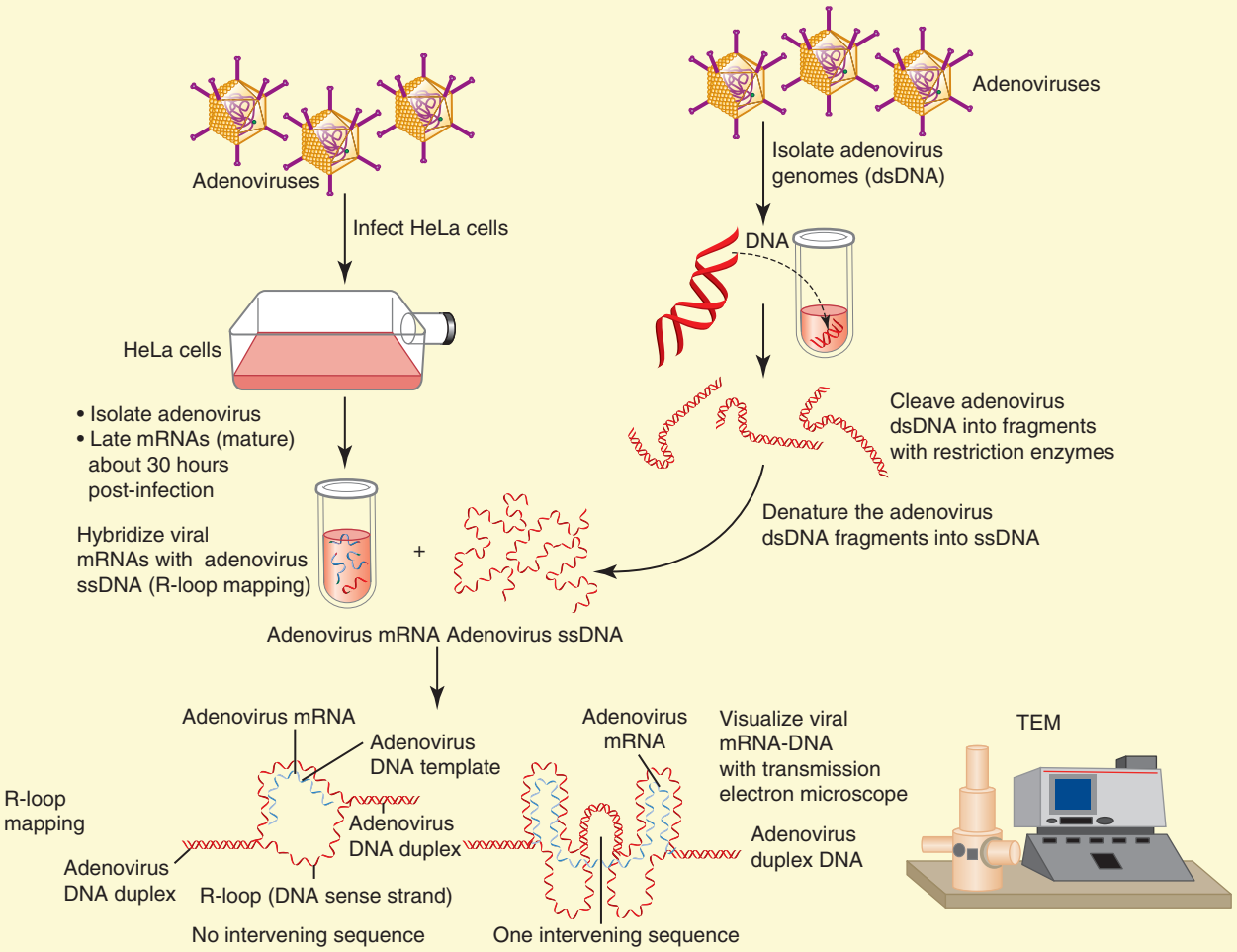

FIGURE 2 Experimental design that led to the discovery of RNA splicing.

intervening nucleotide sequences (now known as introns) were *removed* from the primary pre-mRNA transcripts and flanking sequences (exons) were joined to form the shorter, mature hexon mRNAs that were exported into the cytoplasm for translation (**FIGURE 3C**). In other words, mature viral mRNAs were produced from noncontinuous viral DNA sequences.

Soon after this discovery of splicing, R-loop mapping experiments led by Chambon in 1979 demonstrated that the chicken conalbumin gene contained interrupted noncoding sequences that were missing from the mature mRNAs found in the cytoplasm of cells by R-loop mapping experiments. An explosion of research followed demonstrating similar results for other eucaryotic genes. They used the term *intron* to represent the noncoding sequences embedded within eucaryotic genes. The removal of introns from pre-mRNAs by RNA splicing is an essential function in virtually all eucaryotic organisms. Most genes have multiple introns and are spliced in more than one pattern to yield alternative mRNAs. As a consequence, mutations in splicing signals and splicing machinery have been found to be an underlying defect in a number of human diseases. The lesson on adenovirus 2 mRNAs was an amazing teaching moment that continues to impact many areas of molecular and cellular biology.

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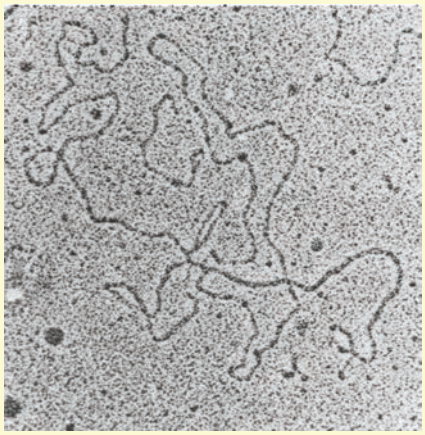
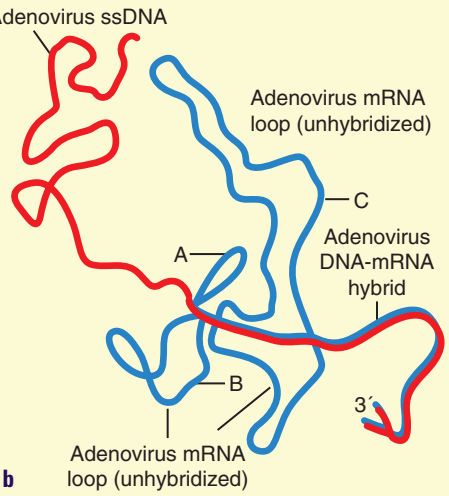
RNA Splicing: A Teachable Moment by Adenovirus 2 (continued)



a

Reprinted from Berget, S. M., et al. 1977. "Spliced segments at the 5′ terminus of adenovirus 2 late mRNA." *PNAS* 74:3171–3175.



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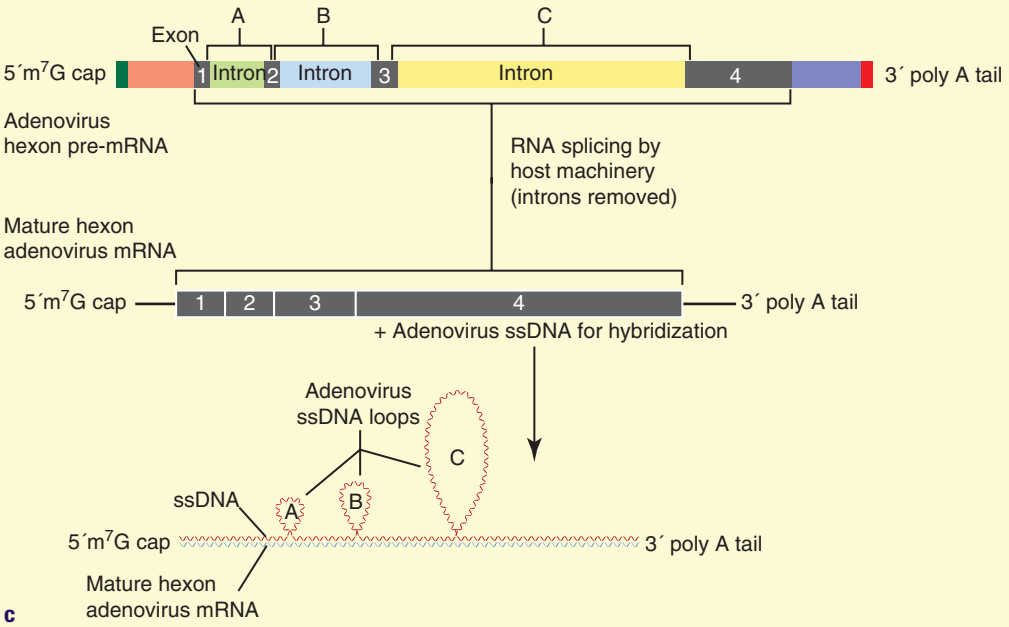
Information from Berget, S. M., et al. 1977. "Spliced segments at the 5′ terminus of adenovirus 2 late mRNA." *PNAS* 74:3171–3175.



c

FIGURE 3 R-loop mapping result demonstrates that the adenovirus 2 gene contains intervening sequences not present in the viral mRNA. **(a)** Transmission electron micrograph of DNA–RNA hybrid. A, B, and C represent unhybridized adenovirus ssDNA. **(b)** Schematic of the electron micrograph shown in (a). **(c)** Schematic illustrating splicing pattern of adenovirus 2 hexon viral coat protein pre-mRNA. The capped, poly(A)-tailed, spliced mRNA was incubated with adenovirus 2 –ssDNA and allowed to hybridize. Diagram shows a linear interpretation of the R-loop mapping results.

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Table 3-1 Eucaryotic Translation Initiation Factors

Factor	Function(s)
eIF1A (eIF4C)	Stimulation of Met-tRNAi and mRNA binding to 40S ribosomes
eIF2	Met-tRNAi binding to 40S ribosomes
eIF2B (GEF)	GDP:GTP exchange on eIF2
eIF2C	Stabilization of ternary complex
eIF3	Ribosome dissociation, stabilization of ternary complex, stimulation of mRNA binding
eIF3A (eIF6)	Ribosome dissociation
Ded1	mRNA binding, RNA helicase
eIF4A	mRNA binding, RNA helicase
eIF4B	mRNA binding, RNA helicase
eIF4E	mRNA binding, cap recognition
eIF4F (CBPII)	mRNA binding, cap recognition, RNA helicase
eIF4G	mRNA binding, anchor protein
eIF4H	mRNA binding
eIF5	Ribosomal subunit joining
eIF5D	Ribosomal subunit joining

Theoretically, for every mRNA, there are six possible reading frames (three in each direction). Some viral transcripts may be translated using multiple ORFs. The mRNA sequence shown in **FIGURE 3-6C** can be read in six reading frames. The three forward reading frames are shown below the sequence, with the translated amino acids listed below each mRNA sequence. Frame 1 starts with the "a," frame 2 with the "t," and frame 3 with the "g." Stop codons are indicated by an "*" in the protein sequence. The only ORF is frame 1 (Figure 3-6C).

Viruses use ingenious strategies to generate many protein products encoded by small genomes that contain a limited number of genes. One of these strategies is **ribosomal frameshifting**, which occurs when the ribosome shifts into another reading frame and then continues translating the mRNA into protein in that new reading frame until an in-frame stop codon is encountered. Hence, certain viruses compress their genetic information by encoding different proteins in overlapping reading frames. Ribosomal frameshifting is very rare in the eucaryotic translation of mRNAs, *but it is a hallmark of the translation of many retrovirus, coronavirus, paramyxovirus, astrovirus, torovirus, and arterivirus mRNAs*. Another strategy that viruses may use to control gene expression is **translational readthrough** (**FIGURE 3-7**), which is a variation of the frameshifting theme. In translational readthrough, a stop translation signal may be ignored.

Cap-Dependent Initiation of Translation

The term **cap-dependent translation** initiation refers to the fact that, with some rare exceptions, all eucaryotic initiation of translation requires the **5′m^{7}Gppp cap** of the cellular mRNA to be present. The structure of the 5′m^{7}Gppp cap is considered to be the signal recognized by the small subunit (40S) of the ribosome to identify the 5′ end of mRNAs because it distinguishes mRNAs from other types of cellular RNAs (e.g., rRNA, tRNAs, and snRNAs). *Some viruses bypass the cap-recognition requirement*. This phenomenon is called **cap-independent translation**. Viruses do this by synthesizing viral mRNAs that contain **internal ribosomal entry sites (IRES)** that structurally allow ribosomes to enter the 5′end of the viral mRNA independently of a cap structure at that end (**FIGURE 3-8**).

Ribosomal Scanning Model

The ribosomal scanning model postulates that a ternary initiation complex is formed (met-tRNAi + eIF-2 + GTP), and that it associates with the small ribosomal subunit (40S). This complex binds/enters the 5′m^{7}Gppp cap of the eucaryotic mRNA, and then migrates and scans linearly down the mRNA, usually stopping at the first AUG it reaches. Marilyn Kozak hypothesized that the 40S ribosomal subunit will scan along the mRNA until it encounters an AUG in the best consensus sequence (usually the first AUG, but not always). After investigating mRNA–ribosomal interactions, she discovered that there was a consensus sequence favored by the ribosome—GCC A/G CC**AUG**(G)—which is now called the **Kozak consensus sequence**. Additional eIFs, the large ribosomal subunit, and ATP also are involved in the initiation of translation (**FIGURE 3-9**). As shown in Table 3-1, many cellular factors are involved in the initiation of protein synthesis. After initiation, elongation and termination are carried out (**FIGURE 3-10**).

Leaky Scanning

Initiation of translation can occur at one or more AUG sites near the 5′ end on a given viral mRNA. The first AUG may not be in a context favorable to the Kozak's consensus sequence. In such cases, the 40S ribosomal subunit may inefficiently initiate translation at the first AUG, but more often the 40S ribosomal subunit will bypass that first AUG and initiate translation farther downstream at an AUG in a better context. This is called **leaky scanning** by the ribosome. It allows multiple viral proteins to be synthesized from a single mRNA. A different protein is generated when the ribosome initiates translation at an alternative AUG.

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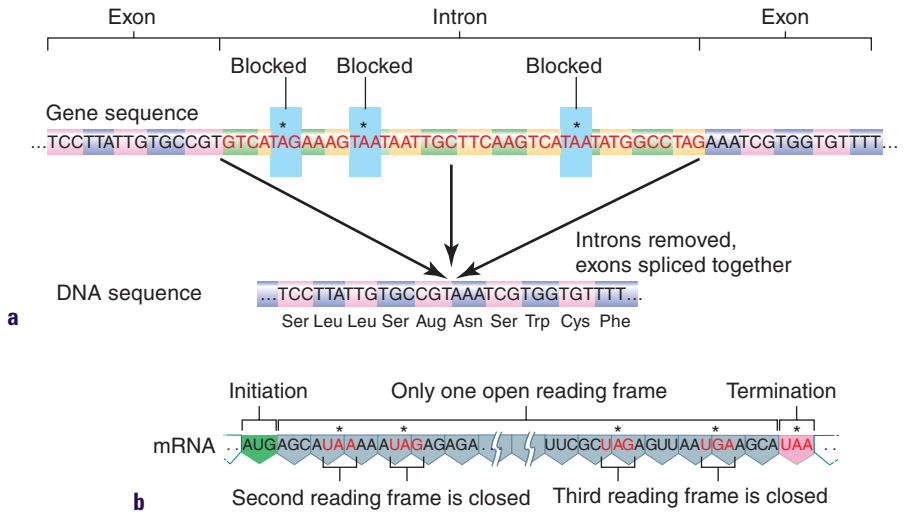

Translation and open reading frames (ORFs)

5′ AUGCCCAAGCUGAAUAGCGUAGAGGGGUUUUCAUCAUUUGAGGACGAUGUAUAA3′ mRNA

ORF

1 aug ccc aag cug aau agc gua gag ggg uuu uca uca uuu gag gac gau gua uaa
MET PRO LYS LEU ASN SER VAL GLU GLY PHE SER SER PHE GLU ASP ASP VAL *

2 ugc cca agc uga aua gcg uag agg ggu uuu cau cau uug agg acg aug uau
CYS PRO SER * ILE ALA * ARG GLY PHE HIS HIS LEU ARG THR MET TYR

3 gcc caa gcu gaa uag cgu aga ggg guu uuc auc auu uga gga cga ugu aua
ALA GLN ALA E * ARG ARG GLY VAL PHE ILE ILE * GLY ARG CYS ILE

c

FIGURE 3-6 **(a)** A continuous ORF is produced when introns are excised from the pre-mRNA. **(b)** In eucaryotes, DNA usually contains one ORF. **(c)** mRNA sequence with the three forward-reading frames shown with the translated amino acids below each mRNA sequence. Stop codons are indicated with an asterisk.

Posttranslational Processing of Proteins

Most eucaryotic proteins undergo some form of modification following translation. **Posttranslational modifications** (such as glycosylation, phosphorylation, and proteolytic cleavage) serve many functions. Phosphorylation of viral proteins is often required for nucleic acid binding, whereas proteolytic cleavage is vital for the maturation and assembly of many viruses. Enveloped viruses have exploited the presence of cell-surface carbohydrates usually associated with glycoproteins, using them as receptors for entry into the host cell.

Why Do All Viruses Use the Host's Protein Synthesis Machinery?

Eucaryotic translation involves more than a dozen initiation, elongation, and termination factors. Most viral genomes do not have the capacity to contain all of the genes necessary to synthesize proteins (Table 3-1). As a result, viruses hijack the host's protein synthesis machinery for the translation of viral mRNAs.

Membranes and Endocytosis

All eucaryotic cells have an external **plasma membrane** that protects the contents of the cell from the outside environment. The cell membrane is semipermeable, allowing specific molecules, such as oxygen, carbon dioxide, and water, to pass through freely and larger molecules such as sugars and amino acids to be more carefully regulated. The plasma membrane is a **phospholipid bilayer** composed of lipids, proteins, and carbohydrates. Scattered throughout the membrane are **integral proteins** that are wholly or partially within the membrane and **peripheral proteins** that lie on the membrane

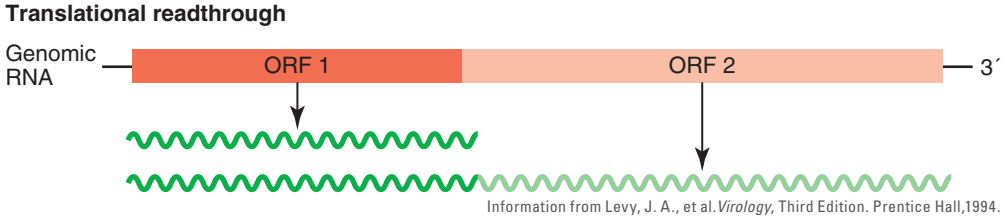

FIGURE 3-7 Translational readthrough generates two overlapping proteins from a single viral RNA.

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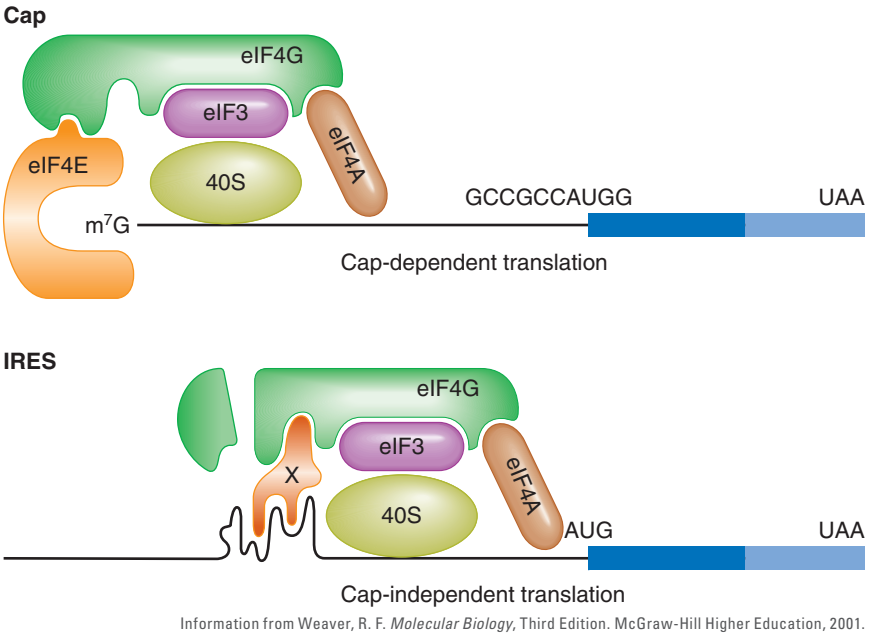
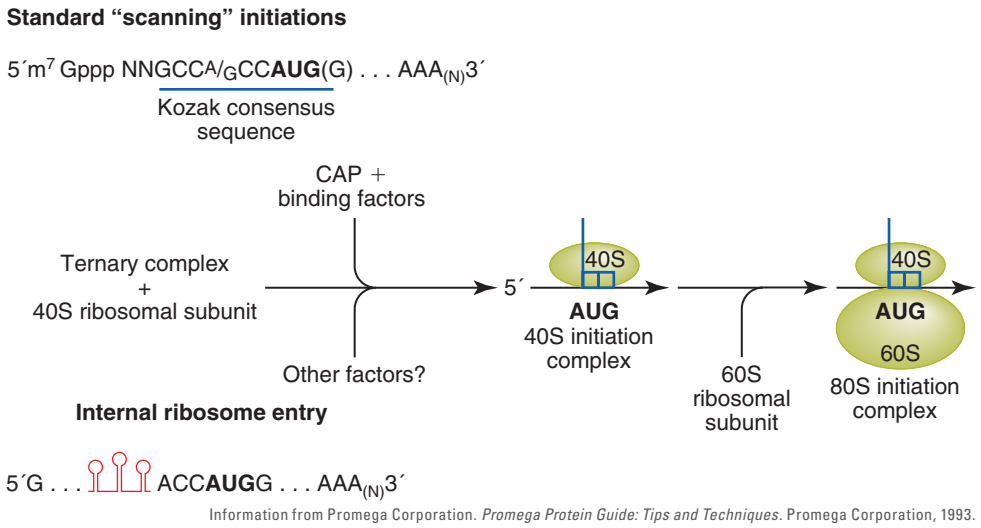


Information from Weaver, R. F. *Molecular Biology*, Third Edition. McGraw-Hill Higher Education, 2001.

FIGURE 3-8 Models of cap-dependent and cap-independent translation.



Information from Promega Corporation. *Promega Protein Guide: Tips and Techniques*. Promega Corporation, 1993.

FIGURE 3-9 Ribosomal scanning model: initiation of translation. The ribosome scans for the best context of the Kozak's consensus sequence.

surface and are bound to the integral proteins. A subclass of integral proteins called **transmembrane proteins** are embedded in the lipid bilayer core, spanning the entire membrane (**FIGURE 3-11**). The outer surface of the membrane is smooth, but the inner surface is covered with many globular proteins. As much as 50% of the mass of a typical cell membrane is composed of proteins.

Membranes are dynamic and fluid, allowing the frequent lateral movement of phospholipids within the membrane. The **fluid mosaic model** refers to the *fluidity* of the lipid bilayer's membrane structure of hydrophilic (polar) heads and hydrophobic tails, along with its collage, or *mosaic*, of intrinsic proteins that are embedded within the membrane. The bilayer functions like channels or conduits through which molecules enter and exit the cell. Integral proteins can freely diffuse laterally throughout the bilayer due to Van der Waals attractions between hydrocarbon tails of the particular phospholipids in the membrane. Proteins do not flip-flop from one side of the membrane to the other and they do not spontaneously rotate because they have an external polar region and internal nonpolar region.

About 25% of all genes encode membrane proteins that are partially embedded or span across the entire membrane. Membrane proteins have diverse functions, such as partitioning the cell into compartments, anchoring cytoskeletal elements, screening molecules for passage into and out of the cell nucleus, connecting cells to each other, and passing signals between cells. The protein composition of each cell is complex. Each type of cell

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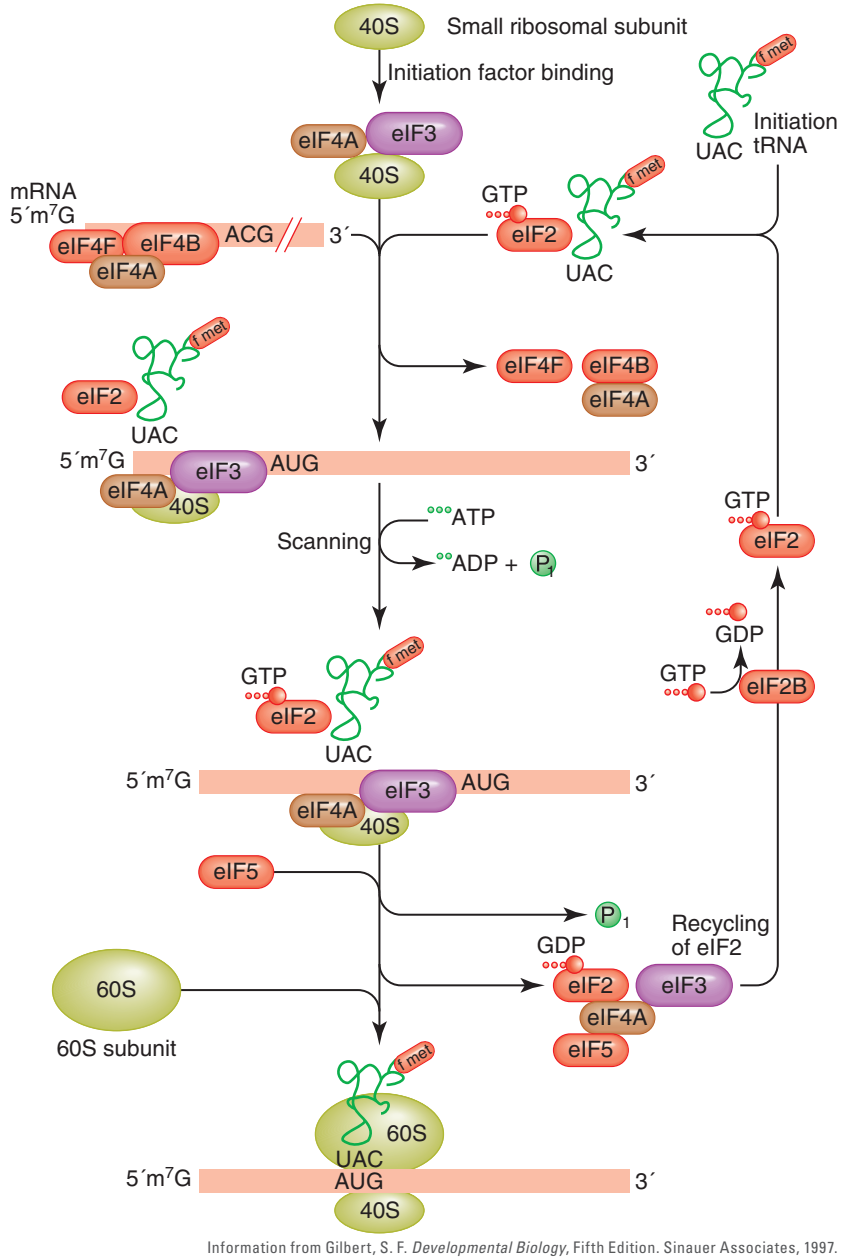

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FIGURE 3-10 Detailed model of eucaryotic initiation of translation. Note the number of eucaryotic initiation factors (eIFs) involved. Their functions are listed in Table 3-1.

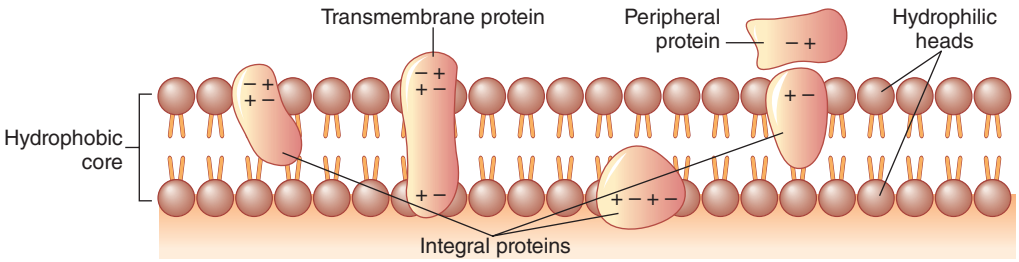

FIGURE 3-11 The structure of a membrane and its associated proteins according to the fluid mosaic model. Notice that the surface of each protein (heavy lines) is in the membrane and the polar region (indicated by + and –) is external. Integral proteins can drift laterally but cannot flip-flop.

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has some unique proteins associated with its membrane, given that the function of a cell is partially defined by its membrane components.

The endoplasmic reticulum, together with the Golgi apparatus, is a major site of *de novo* bulk membrane lipid synthesis. The assembly of membrane lipids requires the synchronous activity of several metabolic pathways that are orchestrated at multiple levels. Viruses do not have the capacity to carry all of the genes involved in **membrane biogenesis**; instead, viruses that are enveloped steal part of the host cell membrane for use as a protective envelope.

An enveloped virus fuses its viral membrane with the membrane of the host cell to infect the cell. The viral entry occurs by the same **endocytic pathways** that cells use to take up fluids, solids, or large particles such as **phagocytosis** ("cell eating"), **pinocytosis** ("cell drinking"), **macropinocytosis** (growth factor–induced, actin-dependent endocytosis), and **receptor-mediated endocytosis** (mainly by clathrin-coated pits in the membrane). A general overview of cellular endocytic and exocytic pathways is shown in **FIGURE 3-12**. The processes differ in the nature of the "cargo," cellular factors involved, and the signals needed for activation. The mechanisms of entry are further divided by those pathways that require the GTPase **dynamin** for **vesicle fission** (the final step in vesicle formation and internalization of virus particles into cells, as opposed to **membrane fusion**, which occurs when the viral/cellular membranes merge during viral entry into a cell) and those that do not. Phagocytosis is used by phagocytic cells to engulf large particles such as bacteria, but larger

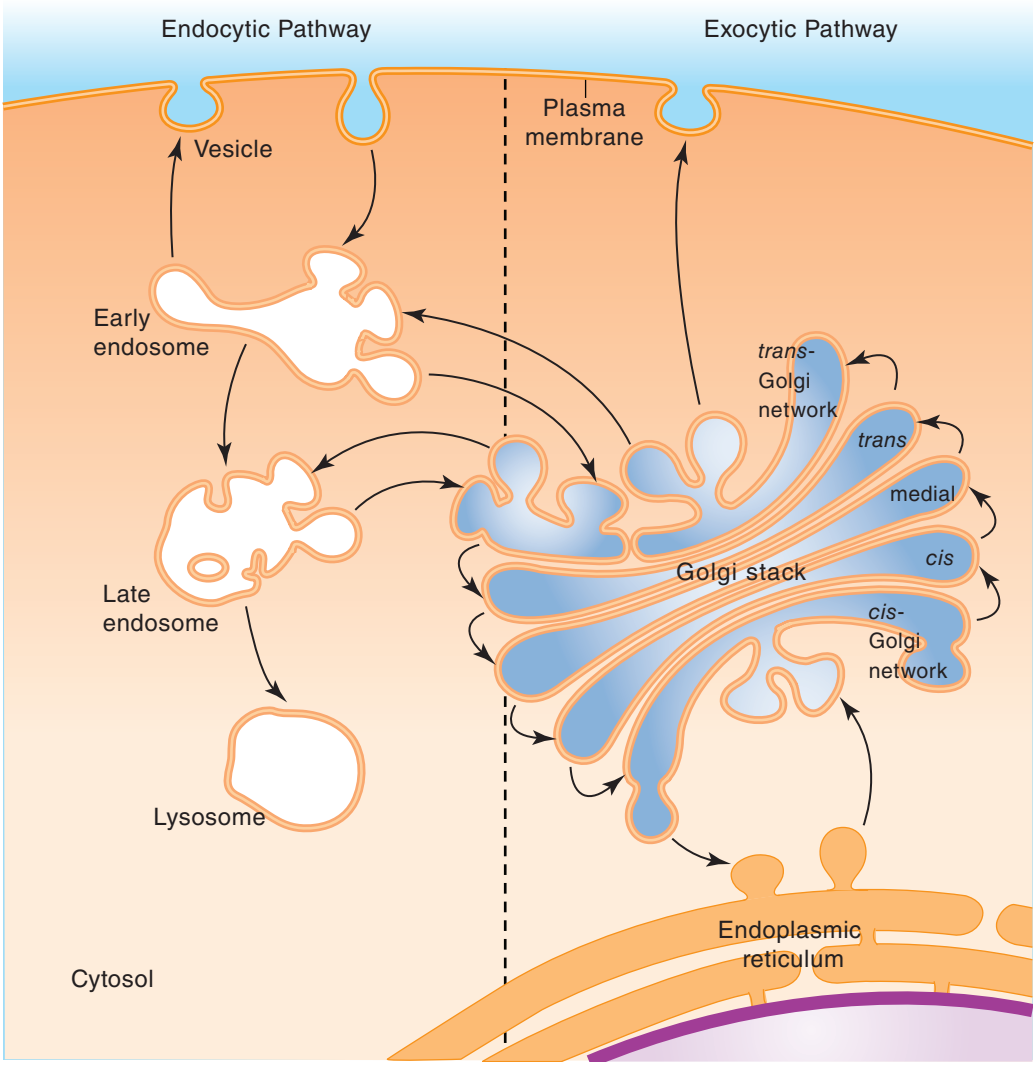

FIGURE 3-12 The two main transport routes of cellular "cargo" are the exocytic pathway (or secretory pathway) and the endocytic pathway, which transports "cargo" out of and into the cells. Vesicles transport internalized material to endosomes, from which other vesicles form to move materials to other compartments. This illustration represents an overview of the endocytic and exocytic pathways used by cells for the movement of proteins in a typical animal or human cell. Almost all of the flow pathways are bidirectional. Viruses are internalized like other cellular "cargo" by endocytic pathways.

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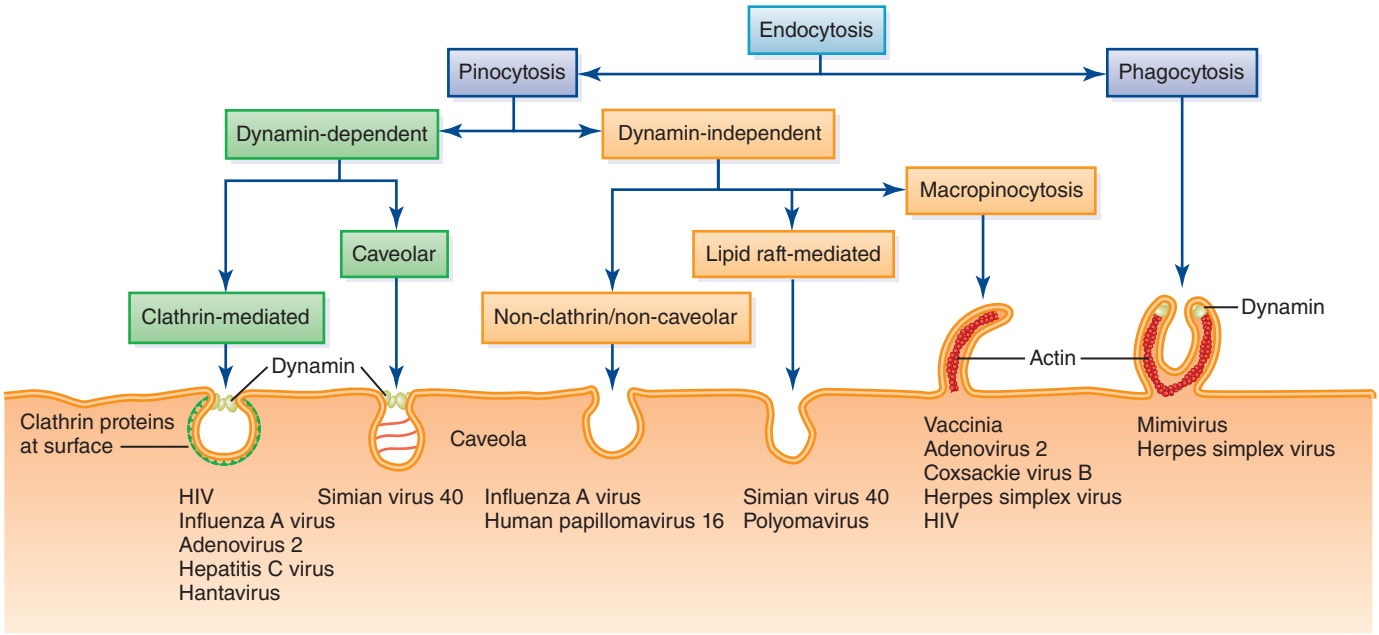


Information from Mercer, J., and Helenius, A. 2009. "Virus entry by macropinocytosis." *Nat Cell Biol* 11:510–520.

FIGURE 3-13 Endocytic pathways used by enveloped viruses to enter host cells. Some viruses can use more than one pathway. The pinocytosis pathways are divided into classes based on whether the GTPase dynamin is required for vesicle fission. Listed below each type of endocytic pathway are examples of viruses that enter cells by endocytosis.

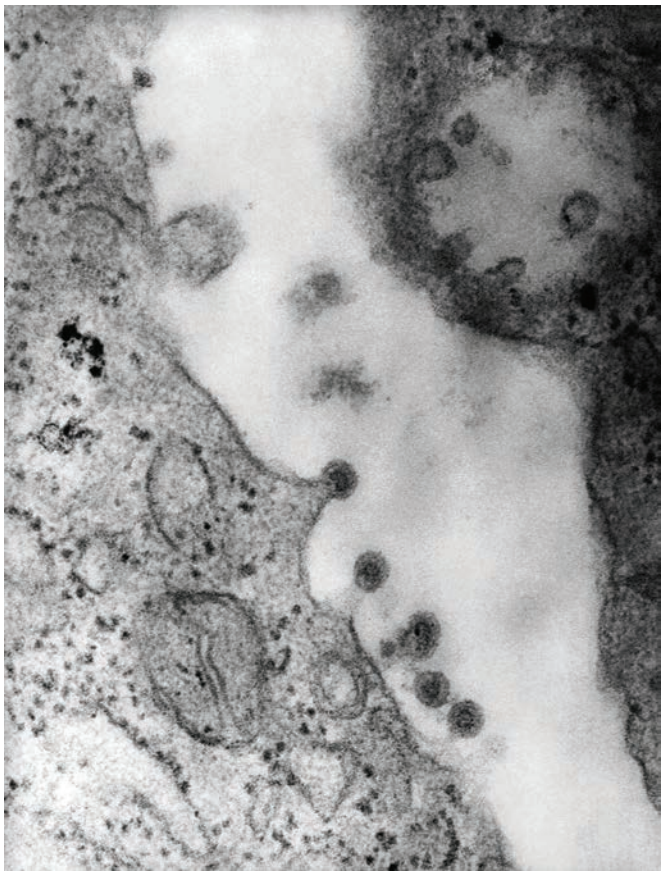
enveloped viruses, such as herpes simplex 1 (which causes cold sores), enters cells through this pathway. *Some viruses enter using more than one endocytic pathway.* In receptor-mediated endocytosis, the virus attaches to a receptor and diffuses along the membrane until it reaches an invagination that is coated on its surface with clathrin proteins. Subsequently the clathrin-coated pit pinches off to form a vesicle, ferrying the virus through the cytoskeletal fibers and transporting it away from the cell surface. **FIGURE 3-13** illustrates the different endocytic pathways used by viruses to enter cells. At the end of their replication cycle, many enveloped viruses assemble within viral factories of the cell and exit through the cellular plasma membrane. As the virus buds from the cell membrane, it provides the newly formed virus envelope (**FIGURE 3-14**). Some viruses may be enveloped more than once, stealing membranes from the host's organelles (e.g., the nuclear envelope or Golgi apparatus).

Intracellular Membranes and Organelles

Eucaryotic cells contain membrane-bound organelles. Viruses recruit organelles for virus replication (e.g., nucleus and mitochondria), uncoating (endoplasmic reticulum–associated degradation pathways and lysosomes), energy (e.g., mitochondria), and assembly (e.g., rough endoplasmic reticulum and Golgi complex).

The Cytoskeleton

Anchored to the interior of the eucaryotic cell's plasma membrane is a complex network of protein filaments or polymers collectively known as the **cytoskeleton**. It is essentially a cellular scaffolding or skeleton made up of



Courtesy of the CDC: Fredrick A. Murphy; Sylvia Whitfield.

FIGURE 3-14 Transmission electron micrograph of rubella virus capsids budding from the infected host cell's surface, producing an enveloped virus (indicated by arrows) as it exits through the plasma membrane. Inside of the virus capsid is the +ssRNA genome. Rubella causes German measles.

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filaments contained within the cytoplasm of the cell. Each filament contains many thousands of identical subunits that are strung together to make a filament. The three types of filaments are **microtubules** (consisting of aggregates of **tubulin**), **actin filaments** (composed of actin subunits), and **intermediate filaments** (bundles of fibrous proteins). The cytoskeleton is involved in cell movement, chromosome separation, and intracellular transport of organelles and provides shape and mechanical strength for the cell. The filaments are in a dynamic, constantly reorganizing state, gaining and losing subunits. Two types of motor proteins drive the movement of microtubules: **kinesins** and **dyneins**.

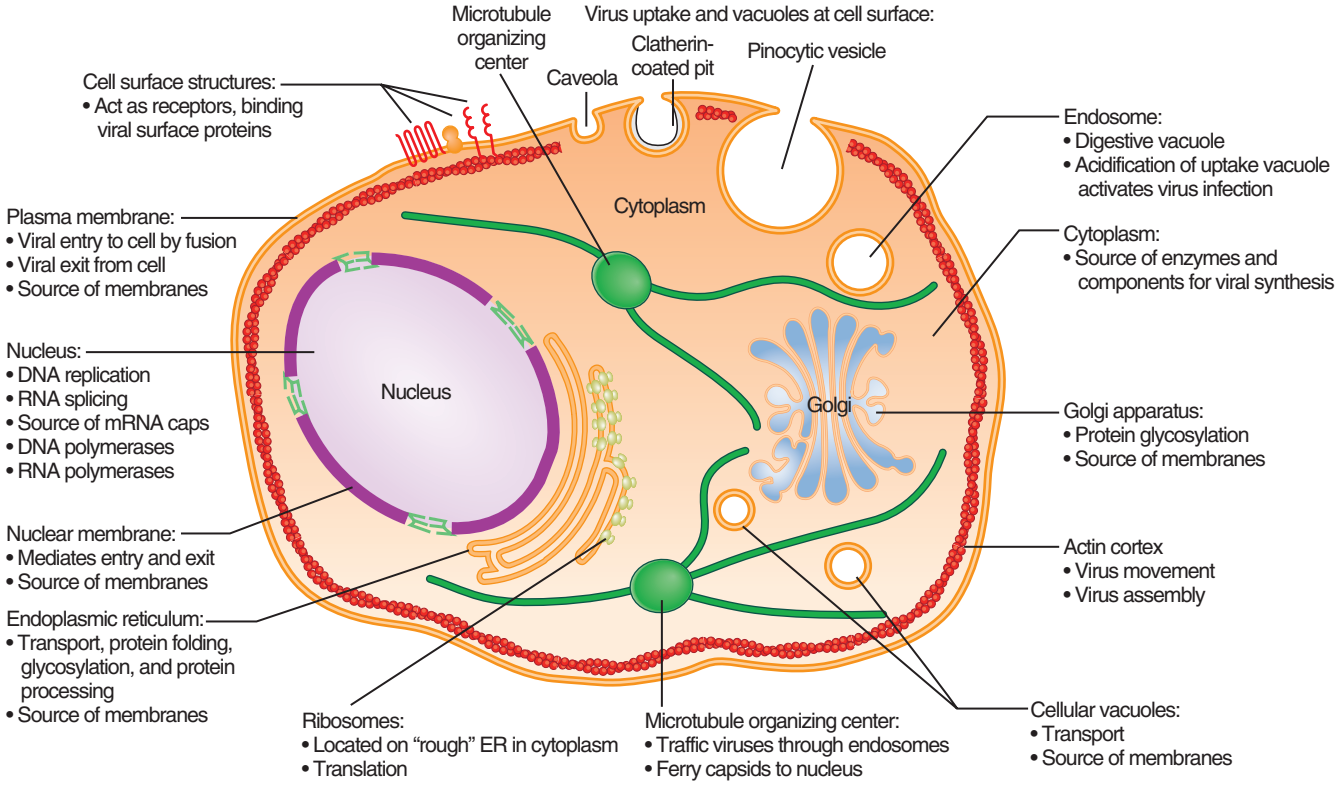
For a virus, it is a pretty large distance from the location where it replicates its genome and the plasma membrane where final assembly and exit from the cell occur. Viruses employ different mechanisms to interact with the cytoskeleton, using the cell's transportation system to move subviral particles through the cytoplasm toward the plasma membrane to the site of final assembly and release. One of the most studied viruses that takes full advantage of the cytoskeleton and its various motors for exit and release is vaccinia virus. Vaccinia virus coordinates polymerization of actin tails, facilitating its cell-to-cell spread. **FIGURE 3-15** illustrates the different cellular compartments or locations, structures, and processes hijacked by viruses for entry, protein synthesis, genome replication, particle assembly, and release.

The Nuclear Envelope

The nuclear envelope is also a significant barrier to viruses that must interact with it and cross the nuclear pore complex. Many viruses must gain access to the DNA polymerases that are present in the nucleus of the host cell.

3.3 Molecular Hurdles of the Host Cell

The cell-free stage when a virus transits to access a new host cell is fraught with danger. It risks damage in the environment and/or when encountering the cell's defense mechanisms. A virus cannot infect every cell type it encounters. It requires contact with and attachment to a host cell that can support its replication. After the attachment "hurdle," the virus particle must penetrate the plasma membrane and *overcome cytoskeletal barriers* such as the **actin cortex** before the journey of the viral nucleocapsid is pampered within the viscous **cytosol** of the cell (**FIGURE 3-16**). The journey through the interior of the cell is crowded with membrane-bound organelles and a meshwork of cytoskeletal fibers. Knowledge of these restrictions can aid in fostering understanding of the unique viral replication strategies that are employed by viruses. Understanding these strategies is central to developing future therapeutics against viruses.



Information from Harper, D. R. *Viruses: Biology, Applications, and Control.* Garland Science, 2012.

FIGURE 3-15 Overview of the host cell locations, structures, and processes hijacked by viruses to enter, move through the cytoplasm, replicate genomes, synthesize viral proteins, assemble new viral particles, and exit the cell.

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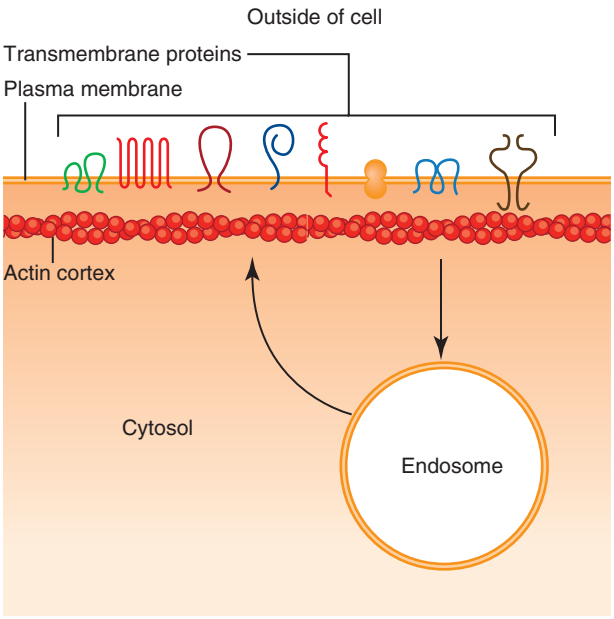

Information from Grove, J., and Marsh, M. 2011. "The cell biology of receptor-mediated virus entry." *J Cell Biol* 195:1071–1082.

FIGURE 3-16 Eucaryotic cells contain barriers that are unwelcoming to viral infection. Viruses overcome these barriers by hijacking endocytic pathways.

Hurdle 1: Receptors and Polymerases

The ability of a virus to replicate inside of a host cell is dependent on the availability of specific cellular proteins. For example, if a host cell lacks a receptor on its surface that the virus can attach to for entry, it will be rejected or restricted from entering and infecting that host cell. Other proteins that host cells may be missing are internal cellular proteins such as transcription or replication factors to support transcription or replication of the viral genome.

Many viruses contain RNA genomes, requiring the need for an **RNA-dependent RNA polymerase** for viral genomic synthesis. *Even though a wide range of host cells contain RNA-dependent RNA polymerases, these cellular polymerases are incapable of replicating viral genomes*. Cellular RNA-dependent RNA polymerases perform different functions. Some function in host defense mechanisms, and others are required for gene regulation. RNA viruses overcome this host cell limitation because their viral genomes contain a gene that encodes virus-specific RNA-dependent RNA polymerases to replicate the viral genome. The viral polymerase gene product is usually packaged along with the viral genome inside of the nucleocapsid.

If a virus containing a DNA genome requires the use of the host cell's DNA polymerase to replicate its genome, it must either infect an undifferentiated dividing cell or have some means of pushing a differentiated cell into the cell cycle so that the DNA polymerase enzymes that it needs for replication will be available. Differentiated cells are not usually cycling through the cell cycle, which means that they will not be producing the DNA polymerases necessary for DNA replication.

Hurdle 2: Actin Remodeling

Actin presents a challenging hurdle to virus entry. One of the most striking observations of infected cells is **actin remodeling** or reorganization. *Actin remodeling affects every stage of a viral replication cycle, from entry through assembly of new virus particles and their subsequent release outside of cells*. Actin filaments are arranged in multiple forms within the cell. **Stress fibers** are large assemblies of actin filaments that span the entire length of the cell. Myosin functions to contract the stress fibers. Underneath the plasma membrane is the loosely organized actin cortex. Actin filaments bundle with other actin filaments to produce cellular extensions or projections such as podosomes (dotlike extensions), lamellipodia (sheetlike extensions), filopodia and microvilli (finger-like protrusions), and large membrane ruffles (**FIGURE 3-17**). Viruses have evolved strategies to engage and manipulate actin in order to gain entry into cells.

Hurdle 3: Ribsomes and Viral mRNA Compatibility

Eucaryotic host translational machinery is restricted to translating **monocistronic RNAs** (monocistronic RNAs code for one protein), and it usually does not recognize internal initiation sites within RNA. Viruses overcome this cellular constraint in at least two different ways. Viruses containing DNA genomes are transcribed into separate RNAs through classic or **alternative splicing (differential)**. Alternative splicing also occurs in eucaryotes. Different proteins are generated from overlapping sequences from a single stretch of DNA. After the DNA is transcribed into pre-mRNA, alternative splicing produces more than one type of mRNA that can be exported into the cytoplasm for translation by the host cell machinery (**FIGURE 3-18**). Viruses that contain **segmented genomes** will generate separate mRNAs through classic or alternative splicing. A segmented viral genome consists of two or more physically separate nucleic acid molecules that are usually packaged within a single virus particle. For example, influenza A viruses contain an RNA genome consisting of eight separate RNAs packaged into the virus particle.

Besides generating separate mRNAs by alternative splicing, viral genomes may be transcribed into a single mRNA encompassing several genes that is then translated into a large precursor **polyprotein**, which is cleaved into individual proteins by viral or cellular proteases (**FIGURE 3-19**). The +ssRNA viruses such as poliovirus and hepatitis C virus are examples of viruses that replicate in this manner.

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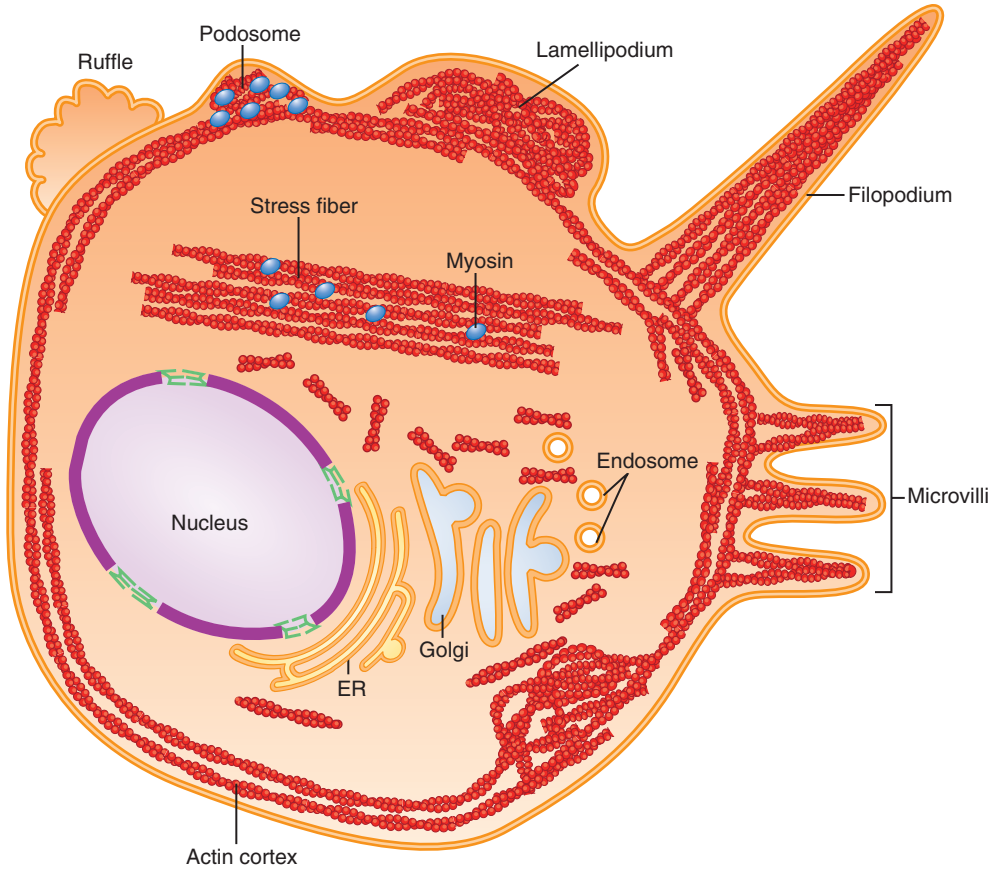

Information from Taylor, M. P., et al. 2011. "Subversion of the actin cytoskeleton during viral infection." *Nat Rev Microbiol* 9:427–439.

FIGURE 3-17 Cellular actin is a physical barrier to virus entry that is present in multiple forms as stress fibers with myosin motors, an actin cortex, podosomes, lamellipodia, filopedia, microvilli, and large membrane ruffles. Actin is manipulated by viruses for entry, movement, and release of new virus particles.

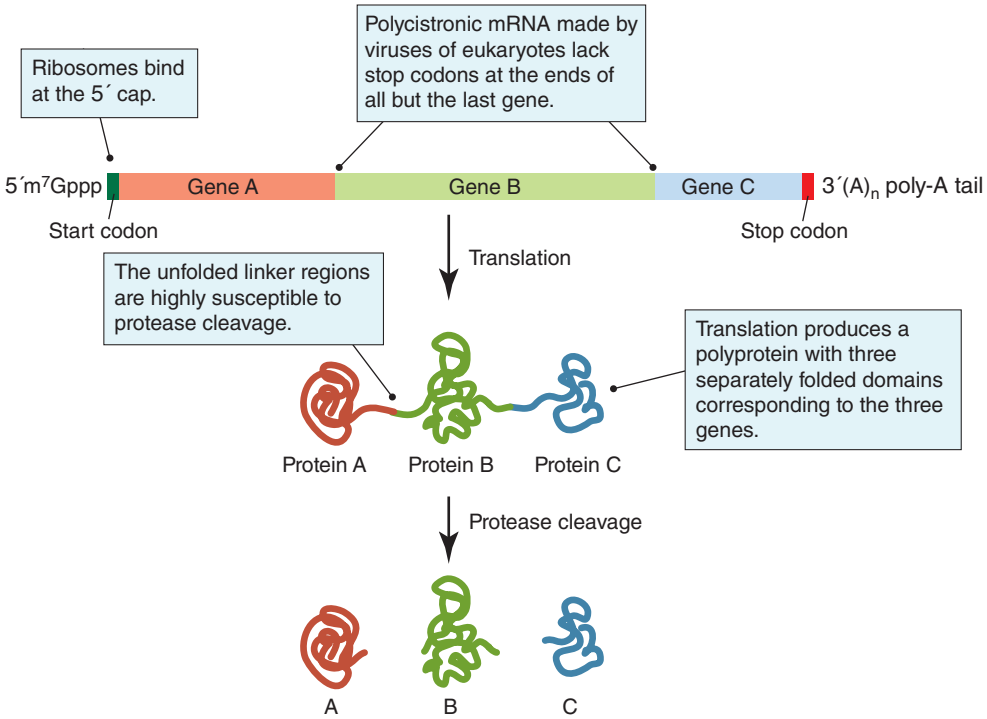

FIGURE 3-18 Eucaryotic cells contain monocistronic mRNAs that are translated into one protein/mRNA. Some viral genomes are transcribed into one precursor RNA that is translated into a large polyprotein. The polyprotein is later cleaved into individual proteins by viral and/or cellular proteases.

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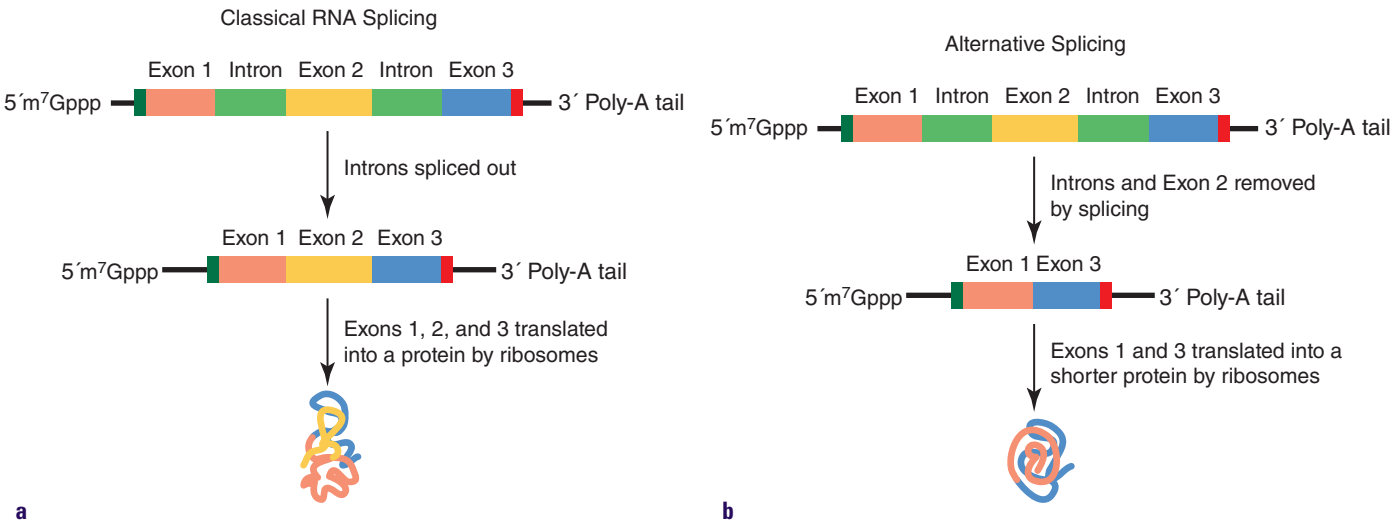

FIGURE 3-19 (a) Introns are removed from the pre-mRNA and the exons are joined in classic RNA splicing reactions. The mature mRNA is exported out of the nucleus into the cytoplasm and translated. **(b)** The same pre-mRNA used in (a) was differentially spliced. The introns along with exon 2 were removed by splicing, resulting in a shorter mRNA that is exported into the cytoplasm and translated into a smaller protein. Alternative splicing generates mRNAs that have different combinations of exons.

Hurdle 4: The Virus–Host Cell mRNA Competition

In an infected cell, the expression of the viral genome is in direct competition with that of the numerous cellular genes. To overcome this hurdle, viruses have evolved ways to produce abundant amounts of their own proteins. Some use strategies that confer a competitive advantage toward viral mRNAs, whereas others preferentially degrade host cell mRNAs.

For example herpes simplex virus 1 (HSV-1) and poxviruses, inhibit cellular translation by using strategies to degrade host mRNAs (and sometimes viral mRNAs as a consequence) following infection, allowing more viral mRNAs to be available to the ribosomes for translation. Influenza viruses cleave and steal host cell mRNA caps that are fused onto the influenza mRNAs, allowing for their preferential translation by the host cell machinery. The decapitated host mRNAs are rendered unstable. The uncapped cellular mRNAs will be degraded by host cell exonucleases, and the decapped host cell mRNAs cannot be translated by the cell's protein synthesis machinery.

3.4 Virus Replication Cycles: One-Step Growth Curves

Viruses depend on host cells for their reproduction, but this requires viruses to overcome certain cellular constraints. Only those viruses that have been able to adapt to their hosts have been able to exist in nature. A single replication cycle of viruses is studied by performing **one-step growth experiments** (sometimes referred to as *single-step growth experiments*). One-step growth experiments provide information about events that occur at each step of the infection cycle (**attachment**, **penetration**, **uncoating**, **replication**, **assembly**, **maturation**, and **release**). Max Delbruck developed one-step growth curve experiments during the 1930s by infecting the bacterium *Escherichia coli* with bacteriophage T4. *E. coli* grows quickly (dividing every 20 minutes) and is lysed (killed) rapidly by **bacteriophage** T4 infection, yielding faster results than traditional methods using animal studies.

In the one-step growth experiments in which *E. coli* was infected with λ bacteriophages, virologists observed a difference in the growth curve representing the production of new virus particles that distinguished it from the growth curve of its bacterial host cells. Shortly after the infection of *E. coli*, the input bacteriophages disappear or are undetectable. The time period in which λ phages are not detected is called the **eclipse phase** (**FIGURE 3-20A**). A **lag phase** occurs during the growth of bacteria in which few bacteria are detected, but there is never a disappearance of bacteria observed during the growth or replication cycle of a typical bacterium such as *E. coli* (**FIGURE 3-20B**). The eclipse phase continues until progeny λ bacteriophages are detectable (anywhere from 11 minutes to an hour or more); this period during which virions are assembled inside of the host cell (cell-associated virus) but not yet released is the **maturation phase**. The **productive phase** of viral infection includes the stages of maturation and release or egress of virus particles (cell-free virus).

Virologists could not study animal and human viruses well in the laboratory before tissue culture methods were developed by John F. Enders, Thomas H. Weller, and Frederic C. Robbins in the late 1940s. Additionally,

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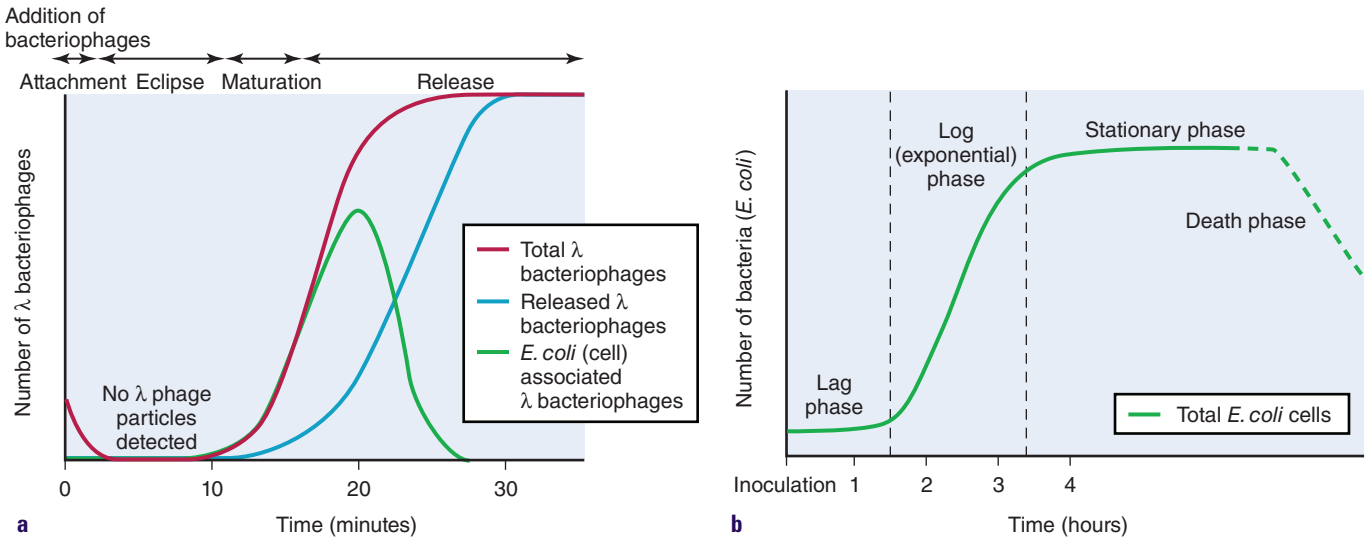

FIGURE 3-20 *E. coli* growth curve versus a one-step growth curve of *E. coli* infected with λ bacteriophages. **(a)** One-step growth curve of bacteriophage λ following infection of *E. coli*. During the eclipse phase, no intact phage particles are detected. Viruses are assembled from preformed "parts" when enough of the preformed parts are made, assembly occurs, and viruses are detectable. **(b)** Growth curve of *E. coli*. Bacterial growth generally proceeds in a series of phases: lag; log (exponential) growth, in which the rate of multiplication is most rapid and constant); stationary (the number of new cells is equal to the number of cells dying); and death phases. Viruses need to be inside of a host cell to replicate.

the development of **Minimal Essential Medium (MEM)** in the 1950s by Harry Eagle was a monumental breakthrough; Eagle's Essential Minimal Medium (EMEM) is still one of the most widely used of all commercially available synthetic cell culture media (**FIGURE 3-21**). Before their work, viruses were injected into eggs or animals and the organs and tissues were analyzed for the pathological signs of viral infection. Only a limited number of viruses, such as influenza virus, could grow in eggs. Experimental animals were difficult to work with and expensive to maintain. Another drawback was that animals were not very permissive to infection with human viruses due to the species barrier and the animal immune response.

Enders, Weller, and Robbins won the 1954 Nobel Prize in Physiology or Medicine for the cultivation of poliovirus in non-nervous tissue cultures (human embryonic skin and muscle cells). Their observations and procedures used to grow viruses *in vitro* contributed to the refinement of tissue culture techniques and played a monumental role in the development of vaccines against poliovirus in the 1950s (**Salk vaccine**) and 1960s (**Sabin vaccine**).

With the advent of cell culture systems, one-step growth experiments were carried out with viruses that infect and replicate in tissue culture cells. Briefly, cell suspensions of tissue culture cells such as monkey kidney cells are allowed to adhere and form monolayers on the bottom of petri dishes. The monolayers of cells are subsequently infected with the virus of choice. The host cells are infected at a high **multiplicity of infection (MOI)** to ensure that every cell of the monolayer is infected

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FIGURE 3-21 Typical reagents used in cultivating mammalian cell culture monolayers in plastic flasks or dishes. *Good* **aseptic technique** *is a necessary skill in maintaining mammalian cell cultures and preparing appropriate media and cell stocks for freezing/storage.* In this photograph, EMEM is a medium being prepared in a vertical laminar flow hood. Premade EMEM can be purchased in powder form for rehydration or as liquid. All media must be sterile. The red medium in the 500-mL glass bottle in the photo contains EMEM that will be supplemented with fetal bovine serum and antibiotics. EMEM is still used today in research laboratories to cultivate many different types of mammalian cells in culture. It contains amino acids, inorganic salts, vitamins, glucose, phenol red (pH indicator), and sodium pyruvate and is usually supplemented with 5–10% fetal bovine serum and antibiotics such as penicillin and streptomycin. Mammalian cell monolayers are washed with sterile phosphate buffered saline (PBS) and treated with trypsin (a protease) to detach the tissue culture cells from the bottom of a flask. A small volume of the cell suspension is removed and diluted with medium. The diluted cell suspension is used to seed a new flask or dishes in order to continue cultivating/passaging cell cultures or to set up experiments involving viral infections in the laboratory.

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simultaneously. The MOI is the average ratio of viruses per cell. For example, an MOI of 10 means that 10 times the number of viruses is used to infect the number of host cells growing in the petri dish. All viruses should be going through the same step in the viral replication cycle at the same time. Infected tissue culture cells are maintained in CO_2 incubators and monitored throughout the course of infection. At various times during the infection, infected cells and/or tissue culture fluid is harvested.

Plaque assays are performed to quantitate the number of intracellular or extracellular virus particles present during that point of infection. **FIGURE 3-22** represents a general laboratory procedure used to perform one-step growth experiments using viruses that infect organisms other than bacteria, such as animals and humans. The same growth curve pattern is observed, but the times for the different phases are longer. For example, the eclipse phase of animal and human viruses lasts hours to several days, depending on the virus.

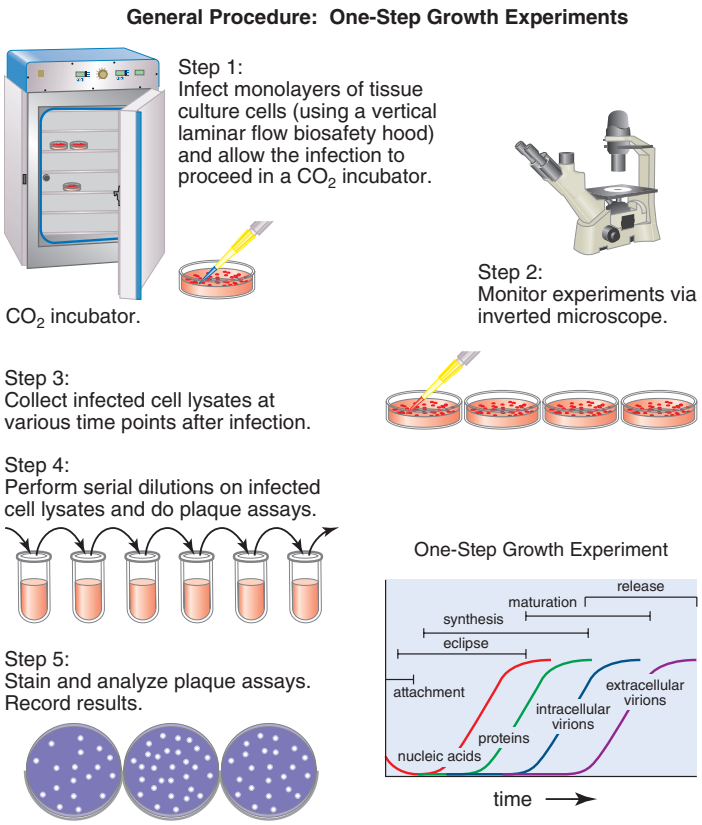

FIGURE 3-22 The diagram briefly outlines the steps involved in performing one-step growth experiments. Step 5 includes a drawing of stained plaque assays. Viral plaques are circular clearings in the cell monolayer where the cells were lysed (killed) by viruses. Purple areas represent stained, healthy, uninfected tissue culture cells. The plaque assay is a quantitative assay used to determine the number of viruses present in a given sample. The results of plaque assays are used to generate a one-step growth curve for a particular virus.

3.5 Key Steps of the Viral Replication Cycle

One-step growth experiments provided the data needed to generate growth curves representing the number of virus particles detected during one replication cycle of a particular virus infecting its target or host cells. By studying growth curves in conjunction with transmission electron microscopy and additional experiments, virologists were able to discern that viral replication involves seven steps: attachment, entry, uncoating, genome replication and gene expression, assembly, maturation, and egress.

Step 1: Attachment (Adsorption)

The first step in the replication cycle of a virus is attachment. The virus must be able to attach to its **host** by entering the "correct" or "target" host cell. The attachment event is electrostatic and does not require any cellular energy. This is a critical step in the viral replication cycle, and a great target for the development of antiviral therapies aimed at *preventing* viral infections. If virus attachment to the host cell is blocked, the infection is prevented. A virus is said to exhibit a **tropism** for a particular cell type when it infects that cell type. In many cases the preferred cell types are a specific population of cells within organs. **TABLE 3-2** lists examples of viruses and their cellular tropism(s). Sometimes viruses also display **species tropism**. For example, poliovirus only infects cells of primates, in contrast to rabies virus, which infects mammals.

Host range is a term that refers to the different types of tissue culture cells or organisms (species) that the virus can infect. The host range may be broad (infecting

Table 3-2 Viral Cell Tropisms

Virus	Cell Type
HIV	CD4+ T lymphocytes, macrophages
Rabies virus	Muscle, neurons
Human papillomavirus	Differentiating keratinocytes
Hepatitis A, B, C viruses	Liver (hepatocytes)
Human herpes simplex virus 1 and 2	Mucoepithelium
Influenza A and B viruses	Respiratory epithelium
Rotavirus	Intestinal epithelium
Norovirus	Intestinal epithelium
Cytomegalovirus	Epithelium, monocytes, lymphocytes
Rhinovirus	Nasal epithelium
Poliovirus	Intestinal epithelium
Epstein-Barr virus	Cell

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many different animal species or cell lines of different species) or narrow. An example of a broad range virus is rabies, for which all mammals have varying susceptibility. **Human immunodeficiency virus (HIV)**, which infects humans and nonhuman primates but causes disease only in humans, falls into the narrow range.

In order to infect cells, attachment proteins located on the viral capsid must be able to bind to **cellular surface receptors**. Cellular receptors are usually proteins, glycoproteins, carbohydrates, or glycolipids. **TABLE 3-3** provides examples of viruses and their cellular receptor(s). *Viruses use these receptors for attachment and*

Table 3-3 Cell Surface Receptors Used by Viruses to Attach and Enter Cells

Virus	Receptor(s)	Reference[a]
Norovirus	Histoblood group antigen (HBGA)	Huang et al., 2003; Lindesmith et al., 2003
Influenza A virus	Sialic acid	Matlin et al., 1981
Poliovirus	CD155	Mendelsohn et al., 1989
Epstein-Barr virus	CD21 and MHC-II	Fingeroth et al., 1984; Li et al., 1997
Adenovirus 2	CAR and integrins α v β 3 and α v β 5	Wickham et al., 1993; Bergelson et al., 1997; Tomko et al., 1997
Hepatitis C virus	CD81 and SR-B1 *claudin-1* and *occludin*	Pileri et al., 1998; Scarselli et al., 2002; Evans et al., 2007; Ploss et al., 2009
HIV	CD4 and coreceptors CCR5 or CXCR4	Dalgleish et al., 1984; Klatzman et al., 1984; Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996
SARS-CoV	Angiotensin-converting enzyme (ACE 2) or liver-SIGN (L-SIGN)	Li et al., 2003; Jeffers et al., 2004
Measles virus	Signaling lymphocyte-activation molecule (SLAM) or Nectin-4	Tatsuo et al., 2000; Noyce et al., 2011
Old World arenaviruses (e.g., Lassa and Lujo viruses)[b]	α-Dystroglycan	Cae et al., 1998
New World arenaviruses (e.g., Junin and Machupo viruses)[c]	Transferrin receptor	Radoshitzky et al., 2007
Hepatitis A virus	TIM-1	Lozach et al., 2011
John Cunningham virus (JCV)	LSTc pentasaccharide	Neu et al., 2010
Ebola virus	TIM-1 and NPC-1	Carette et al., 2011; Cote et al., 2011; Kondratowicz et al., 2011
Rhinovirus (major receptor group)	ICAM-1	Greve et al., 1989; Staunton et al., 1989
Rhinovirus (minor receptor group)	Low-density lipoprotein receptor (LDLR)	Hofer et al., 1994
Coxsackie B virus	Decay-accelerating factor (DAF) and CAR (occludin)	Bergelson et al., 1997; Martino et al., 1998; Coyne et al., 2007
Simian virus 40 (SV40) polyomavirus	GM1	Tsai et al., 2003
Herpes simplex viruses 1 and 2	Nectin-1/2 or herpesvirus entry mediator (HVEM)	Montgomery et al., 1996; Geraghty et al., 1998; Krummenacher, et al., 1998
Rotavirus	Sialic acid and integrins	Yolken et al., 1987; Coulson et al., 1997; Guerrero et al., 2000
Human T cell leukemia virus 1	GLUT-1 or Neuropilin-1	Manel et al., 2003; Jin et al., 2010
Reovirus	Junctional adhesion molecule (JAM)	Barton et al., 2001
Rabies virus	Phospholipids, gangliosides, nAChR,[d] NCAM, p75NTR[d]	Superti et al., 1984; Conti et al., 1986; Superti et al., 1986; Lentz, et al., 1982; Lentz, et al., 1986; Gastka et al., 1996; Burrage, et al., 1985; Thoulouze et al., 1998; Tuffereau et al., 1998; Tuffereau et al., 2001

Factors *italicized* may not directly interact with virus particles but are necessary for viral entry into host cells.

[a]Full citations are listed at the end of the chapter in the section on Primary Literature. Note that the majority of viruses listed are human pathogens.

[b]Old World viruses are found in the Eastern hemisphere in locations such as Europe, Asia, and Africa.

[c]New World viruses are found in the Western hemisphere in locations such as South America and the United States.

[d]There is *no direct evidence in animal models* that these are rabies virus receptors.

Information from Grove, J., and Marsh, M. 2011. "The cell biology of receptor-mediated virus entry." *J Cell Biol* 195:1071–1082.

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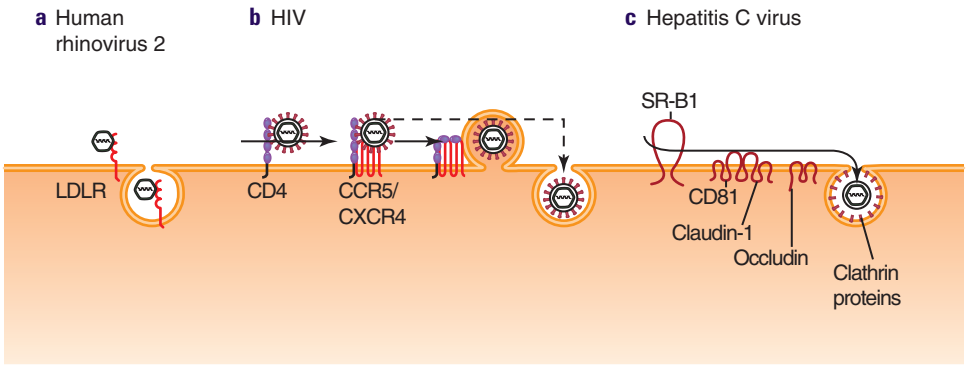

Information from Grove, J., and Marsh, M. 2011. "The cell biology of receptor-mediated virus entry." *J Cell Biol* 195:1071–1082.

FIGURE 3-23 Examples of different cellular receptors initiating virus entry into host cells. **(a)** Human rhinovirus 2 binds to a single receptor, LDLR, meditated by endocytosis. **(b)** CD4 is the primary receptor for HIV, but the virus also requires interaction with a coreceptor, CCR5 or CXCR4. Following coreceptor engagement, fusion occurs and the virus enters by endocytosis. **(c)** Hepatitis C virus requires at least four host cell proteins for entry. To date, it is believed that hepatitis C virus directly interacts with SR-B1 and CD81; however, tight junctions form between claudin-1 and occludin that are indirectly involved. It is not known how the virus is directed to enter by clathrin-coated vesicles.

entry into their hosts. Cell surface receptors play important roles in normal cellular activities.

Some viruses use a single receptor to attach to cells, such as poliovirus, which binds to CD155, or human rhinovirus 2, which binds to the low-density lipoprotein receptor (LDLR; **FIGURE 3-23A**). Other viruses attach to more than one receptor with equal roles, such as SARS-CoV, which binds to angiotensin-converting enzyme (ACE) *or* liver-SIGN. Yet, other viruses have more complex receptor dependency that involves the engagement of at least two different plasma membrane components that are required for viral entry. HIV is the archetypal example. The envelope protein of HIV binds to a primary receptor, CD4, but requires interaction with **coreceptors**, CCR5 or CXCR4, that promote fusion of the viral and host membrane (**FIGURE 3-23B**). Another intriguing example is hepatitis C virus, which requires the expression of four cellular proteins for entry: CD81, SR-B1, claudin, and occludin (**FIGURE 3-23C**).

Receptors are the key to entry into host cells. **VIRUS FILE 3-2** illustrates the classic methods used to discover cell surface receptors. We do not know all of the cellular receptors for every virus; however, imaging technologies are improving and will provide more key insights into the mechanistic details of virus–host interactions at this step of the virus replication cycle.

Step 2: Penetration (Internalization or Entry)

Viruses use many different pathways and strategies to enter cells, some of which are discussed here. After the virus attaches to a cellular receptor, it must break into the cell by crossing intrinsic barriers, including the plasma membrane of the host cell, the meshlike network of the actin cortex, and intracellular membranes. Endosomes are small membrane-bound vesicles or compartments found inside of eucaryotic cells that are involved in sorting "cargo" that has been internalized from the cell surface. Endosomes traffic molecules to lysosomes for degradation or transport molecules to the Golgi apparatus and recycle them back to the plasma membrane. Viruses hijack different cellular endocytic pathways for internalization. (For a review, refer back to Figures 3-12 and 3-13.)

Virus cell surfing is the movement of viruses on the surface of the cells. Viral proteins interact with cellular receptors that are associated with actin filaments just beneath the cell surface. At the cellular actin extension, such as filopedia, virus surfing is driven by the myosin motors within the actin cortex or at the base of filopedia that contract the actin filaments, allowing the virus to move to sites of entry. The movement of the actin cytoskeleton drags the cellular receptor-virion complexes to sites where fusion/endocytosis occurs, stimulating virus entry into cells. Some viruses bind **decay-accelerating factor (DAF)**, which causes cytoskeletal changes as the virus rapidly reaches its specific cellular receptor. For example, during cell surfing coxsackie virus binds to DAF followed by rapid movement to engage the **coxsackie virus and adenovirus receptor** (**CAR**; **FIGURE 3-24**).

Actin-enhanced clathrin-mediated endocytosis is commonly used by viruses for entry. **Clathrin**, which is a large, fibrous protein, is instrumental in the formation of specialized invaginations of the cell membrane called **clathrin-coated pits**. The pits are coated with a lattice-like network of clathrin (dark material) and are located on the cytoplasmic side of the membrane. Activity at the surface of the cell's plasma membrane is dynamic. Membranes are constantly being recycled. The pits are short-lived and soon bud off to form **clathrin-coated vesicles**. The vesicles are for transport. Shortly after formation, the clathrin coat is released and the resultant vesicles are referred to as **endosomes**. The virus particle disassembles (the uncoating step), exposing the viral genome to the

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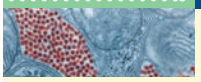
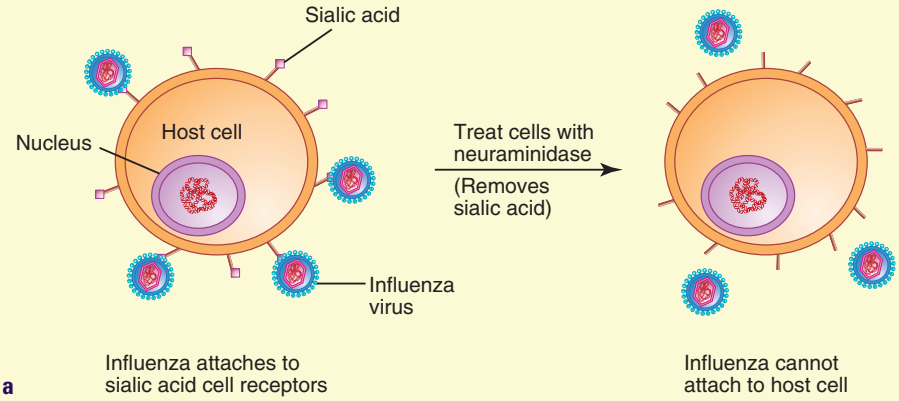
VIRUS FILE 3-2 How Are Cellular Receptors Used for Viral Attachment Discovered?

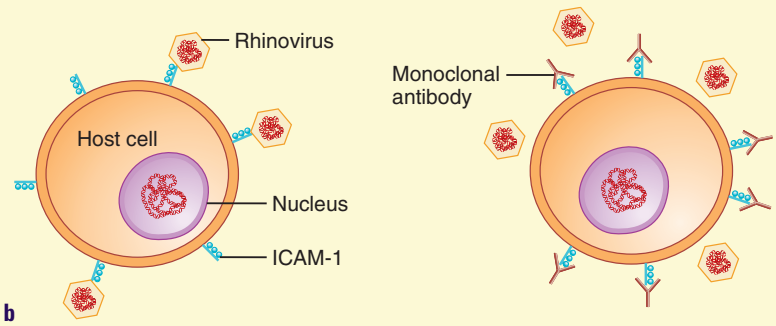


Scientists have developed several techniques to identify the cell surface receptors and coreceptors that viruses attach to in order to initiate infection. These approaches include viral receptor interference studies and genetic techniques. Parts a–c of **FIGURE 1** illustrate the general scheme of the various methods employed.



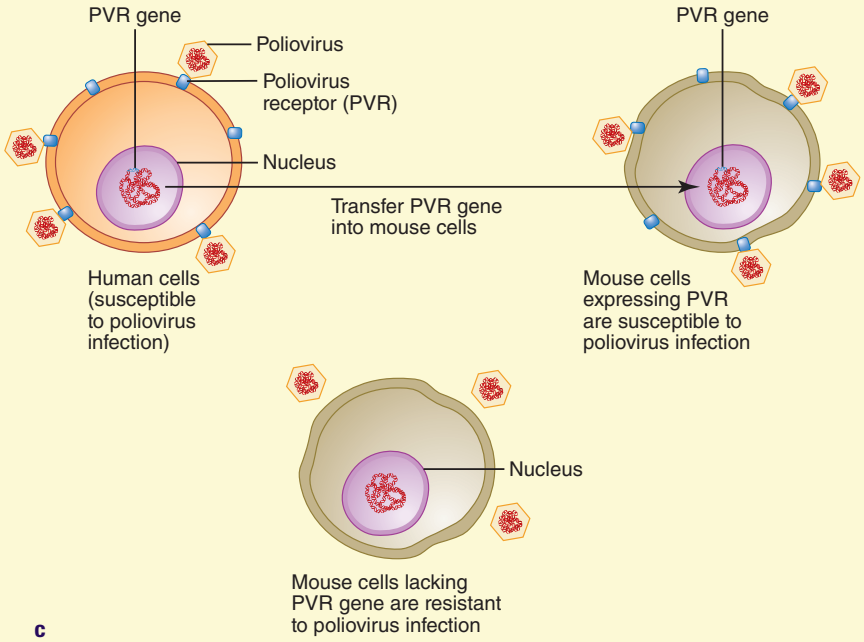
a

Information from Paulson, J. C., and Rogers, G. N. 1987. "Resialylated erythrocytes for assessment of the specificity of sialyloligosaccharide binding-proteins." *Methods in Enzymology* 138:162–168.



b

Information from Staunton, D. E., et al. 1986. "A cell adhesion molecule, I-CAM-1, is the major surface receptor for rhinoviruses." *Cell* 56:849–853.



c

Information from Mendelsohn, C., et al. 1986. "Transformation of human poliovirus receptor gene into mouse cells." *PNAS* 20:7845–7849.

FIGURE 1 Identification of host cell receptors. **(a)** Removal of cell surface receptors. **(b)** Monoclonal antibodies block cell surface receptors. **(c)** Gene-transfer experiments.

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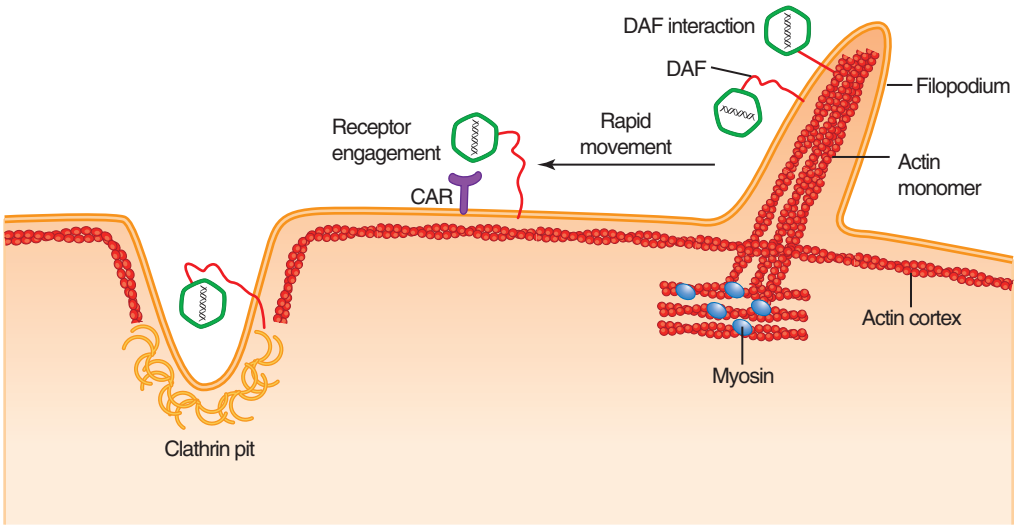

Information from Taylor, M. P., et al. 2011. "Subversion of the actin cytoskeleton during viral infection." *Nat Rev Microbiol* 9:427–439.

FIGURE 3-24 As a virus surfs along the plasma membrane of a cell, the myosin motor at the base of the filopodium pushes the virus to surf down the filopodium where the virus makes contact with DAF. DAF causes a change in the cytoskeleton that allows the virus to rapidly find a receptor to bind to. In this case, the virus is bound to CAR. Actin can also enhance clathrin-mediated endocytosis.

cytoplasm, where it is targeted to the correct location within the cell for genome replication.

The cell surface receptors can directly target viruses for endocytosis by fusion/penetration events at the surface of the host cell or within endosomes by inducing conformational changes in viral capsid proteins. This is referred to as *receptor-mediated endocytosis*. For example, when an enveloped virus attaches to a receptor on the plasma membrane of the cell, the cell is stimulated to engulf the entire virus, forming an endosome. The endosome may fuse with the lysosomes, to form a **multivesicular body (MVB)** that possesses an internal acidic pH. The acidic pH of the MVB causes conformational changes in the viral envelope proteins, facilitating the fusion of the viral and cellular MVB membranes. The viral nucleocapsid is released into the cytoplasm. This mode of viral penetration is referred to as **pH dependent fusion** because it is only at an acidic pH that the fusion between the viral envelope and host cell membrane occurs to release the virus **nucleocapsid** into the cytoplasm (**FIGURE 3-25A**).

Alternatively, cell surface receptors activate specific cellular signaling pathways that facilitate virus entry called **receptor-mediated signaling**. The virus attaches to the cellular receptor on the plasma membrane, initiating a signal within the cell that promotes fusion between the viral and cellular plasma membranes. The activity stimulates cellular actin disassembly or remodeling. The nucleocapsid of the virus is released into the cytoplasm of the cell. The remaining viral envelope remains as a "patch" on the cellular plasma membrane (**FIGURE 3-25B**). The mode of plasma membrane penetration in this alternative scenario is **pH independent fusion**.

Step 3: Uncoating (Disassembly and Localization)

The first problem any virus faces after breaking into the cell is how to transport the viral genome to the site of genome replication, which may be the nucleus, some distance from its point of entry. The virus must find its way through cellular membranes and a crowded cytoplasm. Furthermore, the encapsidated genome within the virion is inactive and must be freed from its protective coat. This step, uncoating, refers to the removal or degradation of the capsid, thereby releasing the genome into the host cell. Enveloped viruses such as influenza A virus fuse their lipid membrane with the plasma membrane or an endosome that uncoats/dissociates the genome from the capsid, and subsequently the viral genome is transported through the nuclear pore complex (**FIGURE 3-26**).

The capsids of naked viruses are cemented together with proteins interlinked by divalent cations or disulfides that must be broken in a stepwise manner for uncoating to occur. Naked viruses undergo uncoating through receptor, chemical, or mechanical cues. Chemical cues occur within specific compartments of the cell. Examples include low pH (H^+ ions) in endosomes; oxidoreductases and thioloxidases involved in protein folding in the endoplasmic reticulum; quality-control factors in the lumen associated with the degradation and export of misfolded proteins using the **endoplasmic reticulum-associated degradation** (**ERAD**) machinery; or low calcium ion (Ca^{2+}) concentrations in the cytosol. Mechanical cues include the use of molecular motors such as the myosin motors of actin or kinesin motors of

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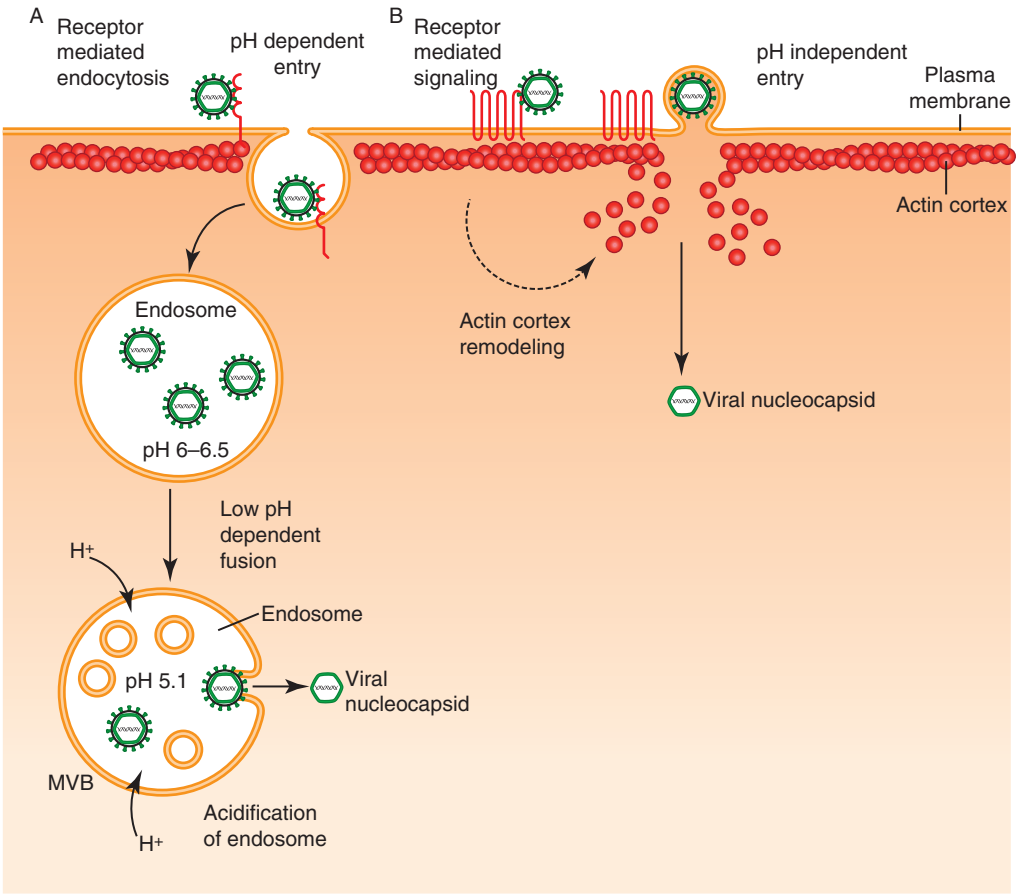

Information from Grove, J., and Marsh, M. 2011. "The cell biology of receptor-mediated virus entry." *J Cell Biol* 195:1071–1082.

FIGURE 3-25 Virus entry strategies. **(a)** The enveloped virus enters by receptor-mediated endocytosis followed by *pH dependent* fusion of the virus and MVB, releasing the nucleocapsid of the virus into the cytoplasm. **(b)** The enveloped virus and plasma membrane undergo *pH independent* fusion coupled with receptor-mediated signaling and remodeling of the cellular actin cortex.

microtubules that immobilize host proteins interacting with the viral capsid, creating mechanical strain that leads to the disassembly or uncoating of the capsid.

Receptor binding causes conformational changes that impact the uncoating of picornaviruses. Simian virus 40 and mouse polyomavirus do not uncoat upon binding to receptors; instead the virus particles travel within a caveosome via the motor action of microtubules to the endoplasmic reticulum (ER). Host factors and chemical cues within the ER catalyze partial disruption of disulfide bonds, causing structural changes within the virus capsid. The virus particles escape the ER using the ERAD pathway, entering the cytosol with its reducing conditions and low Ca^{2+}, causing the capsids to undergo more destabilization and uncoating. Other factors mediate the import of the virus genome into the nucleus, the site of its replication (**FIGURE 3-27**).

Human adenovirus 2 and adenovirus 5 interact with the CAR by fiber proteins followed by engagement with integrin receptors. Integrin receptors are immobile, whereas CAR will drift. The drifting mechanism is dependent upon the myosin motors of actin. The drifting by CAR induces conformational changes that weaken the capsid, resulting in the loss of the adenovirus fibers. Subsequently the spikeless virus capsid enters the cell within a clathrin-coated vesicle by endocytosis. The capsid is transported to endosomes. Within the late endosome, the capsid undergoes more structural changes and escapes into the cytosol. The weakened capsid docks on the microtubules and its associated motors, which then move the capsid to the nuclear pore complex where it docks again. The motor action disrupts the capsid such that the viral genome can be imported to its site of replication into the nucleus via the **nuclear pore complex** (**FIGURE 3-28**). When the nucleocapsid is uncoated, infectious particles are no longer detected in one-step growth experiments. This is the start of the eclipse phase, which continues until new virions are assembled.

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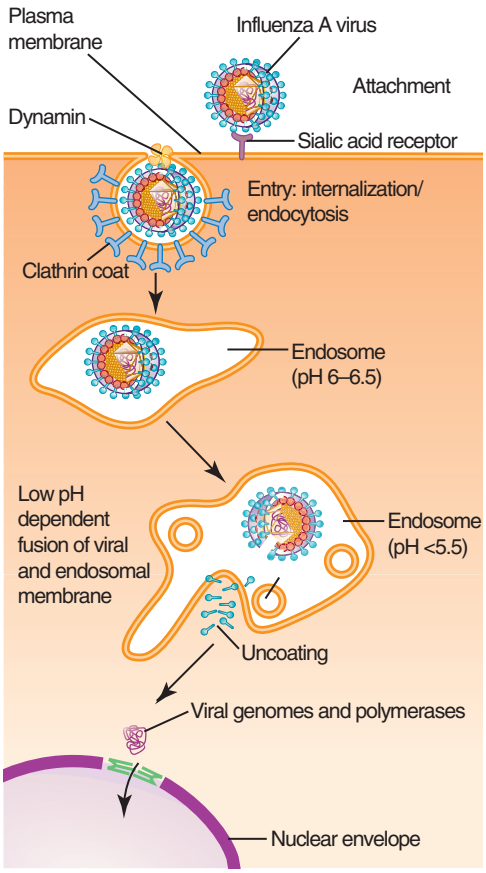
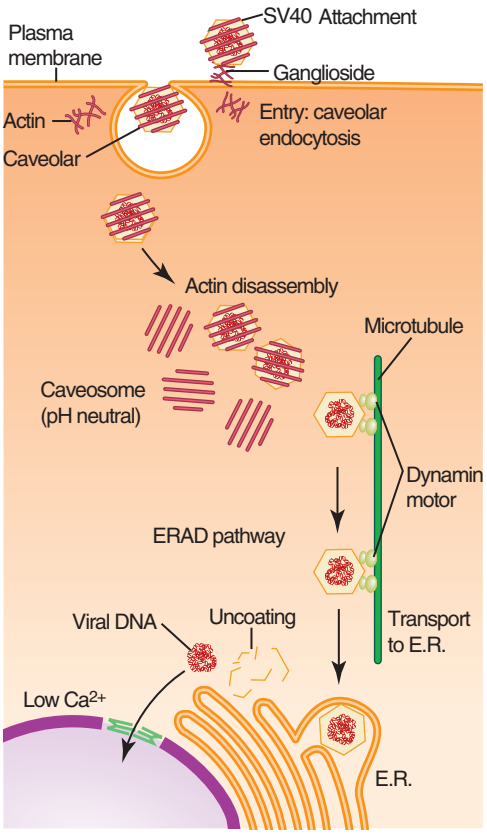


Information from Smith, A. E., and Helenius, A. 2004. "How viruses enter animal cells." *Science* 304:237–242.

FIGURE 3-26 Entry and uncoating of influenza A virus. Influenza A virus is enveloped and contains an ssRNA genome of negative polarity that is segmented (see section on replication). The surface protein (hemagglutinin; HA) of influenza A virus binds to sialic acid moieties of glycoproteins or glycolipids present on epithelial cells of its host. The influenza A virus enters the host cell after it is internalized by a clathrin-coated pit and transported by the early endosomes to the late endosomes in which the low pH activates fusion of the viral and cellular envelope, resulting in virus uncoating and release of the viral genome. The viral genome and associated proteins move through the nuclear pores and into the nucleus of the host cell for replication.



Information from Smith, A. E., and Helenius, A. 2004. "How viruses enter animal cells." *Science* 304:237–242.

FIGURE 3-27 Entry and uncoating of simian virus 40 (SV40). SV40 is a naked virus that contains a circular dsDNA genome that replicates within the nucleus of its host cell. SV40 attaches to gangliosides in the plasma membrane and enters the cell by caveolar endocytosis. The virus travels along the microtubules driven by dynein motors to the endoplasmic reticulum (ER). Within the ER, the integrity of the virus capsid is compromised. The virus escapes from the ER into the cytosol using the ERAD pathway. The low Ca^{2+} concentration in the cytosol facilitates uncoating. The SV40 dsDNA genome enters the nucleus through the nuclear pores.

Step 4: Genome Replication and Gene Expression

When viruses infect cells, two important and separate events must occur:

1. *Viral structural proteins and enzymes must be produced.*
2. *The virus genome must be replicated.*

The genome of a virus consists of DNA or RNA, which may be single stranded (ss) or double stranded (ds) and linear or circular. The entire genome may occupy either one nucleic acid molecule (**monopartite** or **nonsegmented genome**) or several nucleic acid molecules (**multipartite** or segmented genome). The different genome types are classified into seven groups based on the strategies used to generate viral mRNAs for translation. This grouping is called the **Baltimore classification** system (**FIGURE 3-29**). Viruses build **replication organelles**, which recruit cell and viral components within a macrostructure in which viruses assemble and mature.

DNA Viruses

Most DNA viruses replicate their genomes in the nucleus and utilize the host's DNA replication and RNA transcriptional and splicing machinery. In doing so, the viral genome must traverse the nuclear membrane to utilize the aforementioned cellular machinery.

dsDNA Viruses Replication of many DNA viruses involves strategies that are familiar in cell biology:

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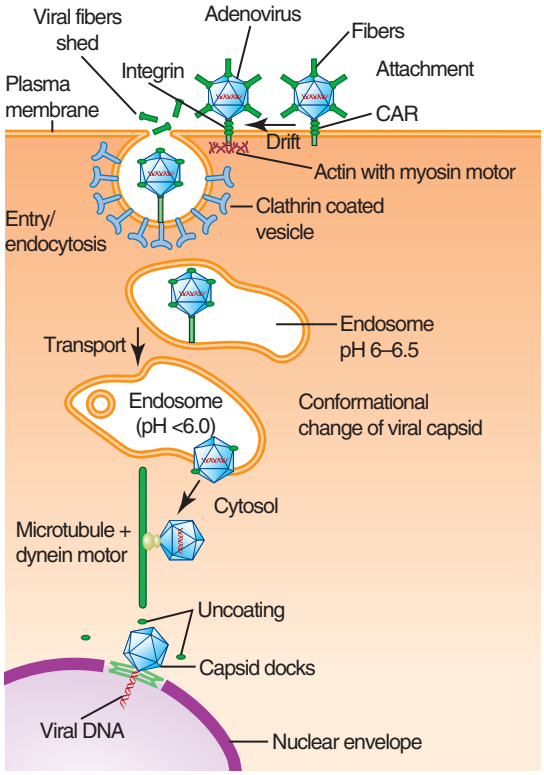

Information from Smith, A. E., and Helenius, A. 2004. "How viruses enter animal cells." *Science* 304:237–242.

FIGURE 3-28 Entry and uncoating of adenovirus 2. Adenovirus 2 is a naked virus that contains a dsDNA genome. The virus is an icosahedral capsid with protruding fibers. The viral fibers attach to the CAR receptor. The myosin motors of actin move the adenovirus attached to CAR as it drifts toward nearby fixed (immobile) integrins within the plasma membrane. The mechanical strain dissociates the fibers from the capsid. The spikeless capsid is internalized within a cathrin-coated vesicle via endocytosis and transported to the endosomes. Within the late endosome, the capsid structure undergoes more structural changes, allowing the capsid to escape into the cytosol of the cell. The weakened capsid docks on the microtubules, moving the capsid to the nuclear pore complex, where it docks again and undergoes the final step in uncoating. The dsDNA genome enters the nucleus, where it will undergo replication.

carrying out DNA replication and mRNA transcription from dsDNA in the cytoplasm of cells. Viral proteins are translated from the monocistronic mRNAs generated through the transcription of viral mRNAs. Most dsDNA viruses use the host cell's RNA polymerase II, but some of the larger viruses bring their own RNA polymerase. Most dsDNA viruses utilize the cell's nucleus for DNA replication and RNA transcription. A simplified illustration of the replication cycle of an enveloped dsDNA virus is shown in **FIGURE 3-30**.

Poxviruses differ from the other dsDNA virus families in that they replicate solely in the cytoplasm. The poxviruses carry their own DNA-dependent DNA polymerase (to replicate the viral dsDNA genome) within the virus particle. The genomes of poxviruses are large (ranging from 130 to 230 kilobase pairs [kbp], or roughly 130–230 genes), allowing the virions to be fully equipped with the genes to make them independent of the host's nuclear enzymes and machinery. The monocistronic mRNAs are transcribed directly from the viral dsDNA. Like poxviruses, Mimiviruses also possess their own transcriptional apparatus (see **VIRUS FILE 3-3**).

ssDNA Viruses The ssDNA viruses must convert their linear ssDNA to a dsDNA form. *The host cell does not contain any DNA-dependent RNA polymerases to convert ssDNA into mRNA*. There are cellular DNA polymerases that can convert viral ssDNA into dsDNA, followed by cellular RNA polymerase II that can transcribe the viral dsDNA intermediate into viral mRNAs in the nucleus. The cellular RNA splicing machinery is also used in the production of mature viral mRNAs. Each virion packages one of the two DNA strands, but some viruses package both strands, resulting in two different kinds of virions that are identical except for the polarity of the ssDNA.

ss/dsDNA Viruses (Using an RNA Intermediate) Hepadnavirus genome replication uses a very unique and somewhat complicated mechanism. Hepadnaviruses contain *dsDNA* genomes and infect and cause *hepatitis* in woodchucks, ground squirrels, chipmunks, ducks, geese, chimps, gibbons, orangutans, and humans. Hence, the use of the term *hepadnavirus* to describe this group of viruses. Hepatitis B virus (HBV) specifically infects humans. Interestingly, cells infected with HBV produce different forms of virus particles. Electron microscopy of partially purified virus particle preparations reveal three types of particles: a 42- to 47-nm mature spherical virus particle (known as a **Dane particle**, after its discoverer); 22-nm spherical particles, which are found in 10,000- to 100,000-fold excess over the Dane particle; and filamentous particles that are 22 nm in diameter and of varying lengths. All three forms contain the same surface protein, called the *hepatitis B surface antigen* (HBsAg). The Dane particle is the only infectious particle of HBV. The 22-nm spheres and filaments do not contain nucleic acid (**FIGURE 3-31**).

The genome of the Dane particles consists of a 3.2-kb linear DNA that is arranged in a relaxed circle. Some parts of the genome are dsDNA, whereas others consist of ssDNA regions or gaps. This partially duplexed DNA consists of a full-length (–) sense ssDNA (–ssDNA) and a shorter-length (+) sense ssDNA (+ssDNA). As a result, the gapped regions contain only –ssDNA. After the HBV has entered its host cell and the virus is partially uncoated, the partial dsDNA genome of the Dane particle migrates to the nucleus, where it is completed or repaired by a viral reverse transcriptase. The dsDNA enters the nucleus, and its ends are ligated together by cellular enzymes, forming a circular episome. The term **episome** applies to a viral genome that is maintained in cells by autonomous replication. Next, the repaired viral dsDNA

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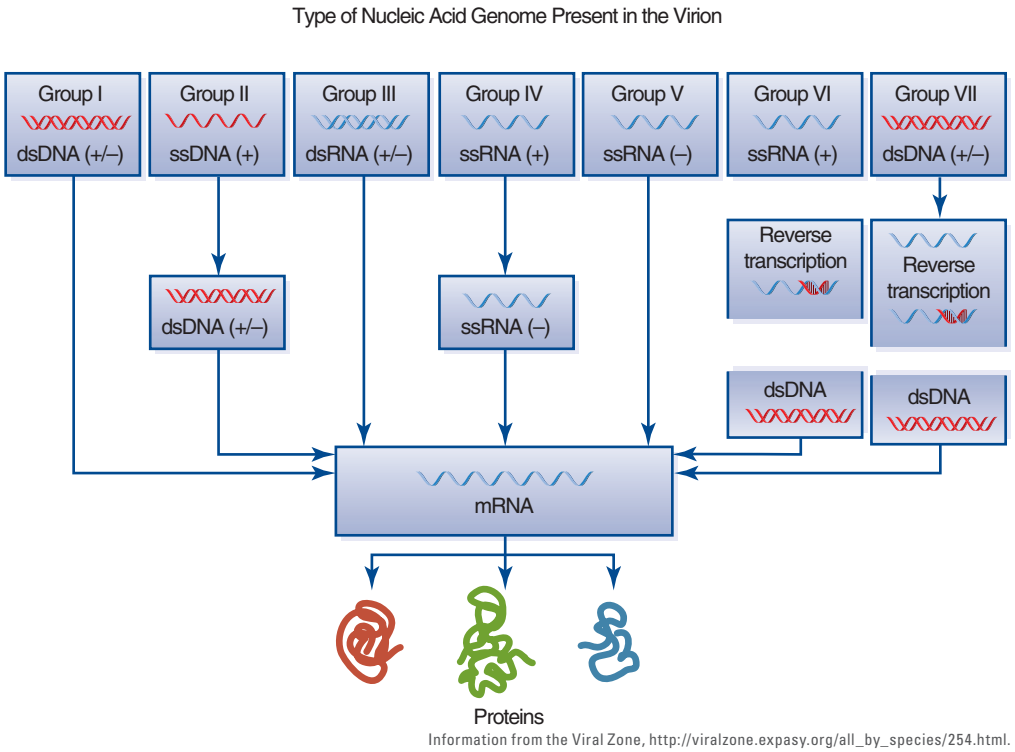

FIGURE 3-29 Baltimore classification of viruses. There are seven groups of viruses based on the type of genome and strategy used to synthesize viral mRNAs. The genomes are composed of single- or double-stranded RNA synthesized by RNA polymerases or single- or double-stranded DNA that are synthesized by DNA polymerases. Positive-sense ssRNA (+ssRNA) is the equivalent of an mRNA translated by the host cell protein synthesis machinery. Negative sense ssRNA (–ssRNA) is an antisense RNA that must be converted to a +ssRNA for translation by the host cell ribosomes.

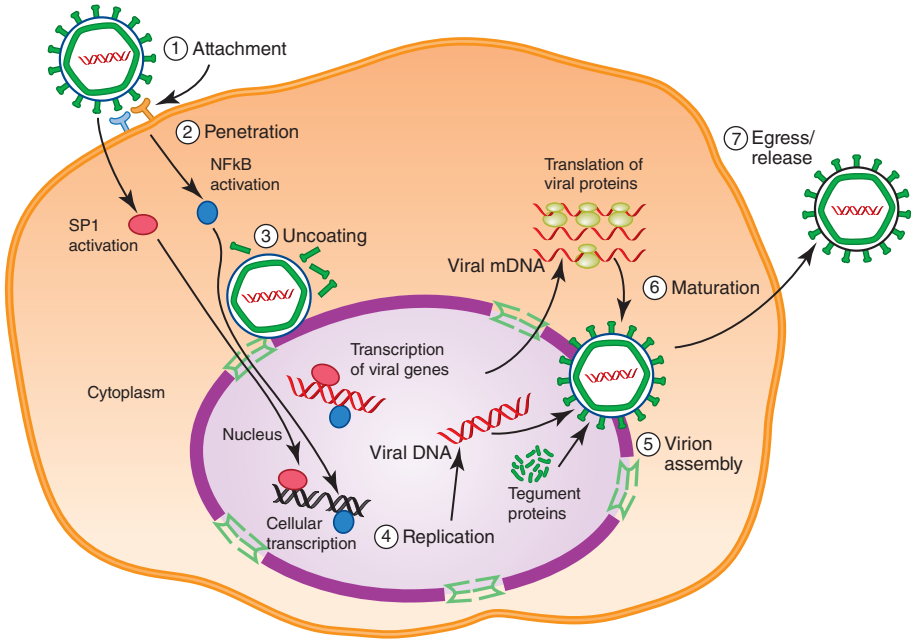

FIGURE 3-30 Simplified illustration of an example of a replication cycle of a dsDNA virus (cytomegalovirus). Cytomegalovirus is a member of the *Herpesviridae* family. The gH and gB glycoproteins present on the outside of the cytomegalovirus particle bind to the cellular receptors. The attachment event triggers cellular transcription factors SP1 and NF-κB to migrate to the host cell nucleus. After the penetration and uncoating step, the viral cytomegalovirus dsDNA is released and enters the host cell nucleus where the DNA is replicated and transcribed with the help of host cell transcription factors SP1 and NF-κB. Viral mRNAs are exported into the cytoplasm where they are translated by the cellular machinery. Viral dsDNA and viral and cellular proteins are packaged into the virion. Several models have been developed regarding the envelopment of the virion. The virion may be enveloped as it buds out of the nuclear membrane or de-enveloped and re-enveloped at the Golgi membranes following virion release.

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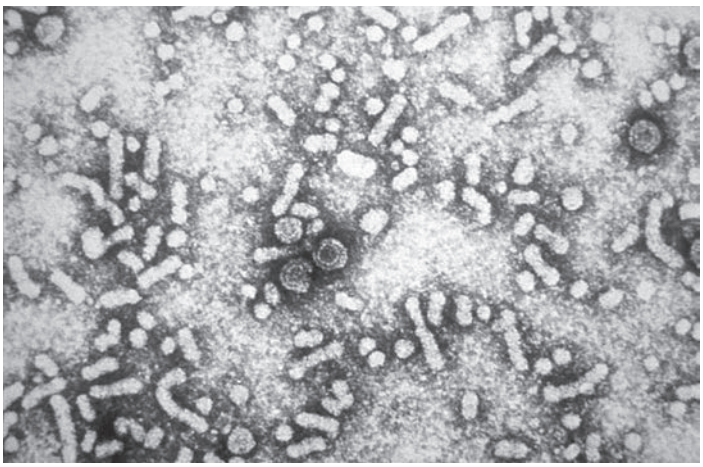

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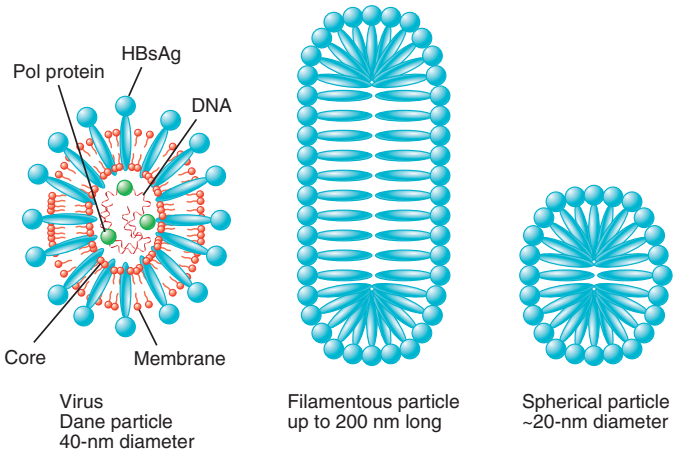
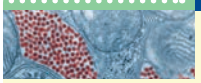


b Information from University of South Carolina, School of Medicine. "Virology: Hepatitis Viruses." Microbiology and Immunology. University of South Carolina, 2008. http://pathmicro.med.sc.edu/virol/hepatitis-virus.htm.

FIGURE 3-31 **(a)** HBV infection results in the formation of three different types of virus particles: 42- to 47-nm intact infectious Dane particles, 22-nm spheres, and 22-nm filaments of varying lengths. **(b)** Illustration depicting the different forms of HBV particles. Mature hepatitis B virus Dane particles contain dsDNA with associated protein, but their mode of replication is different from the other dsDNA viruses and their replication strategy.



VIRUS FILE 3-3 Unraveling the Replication Cycle of Mimivirus

The discovery of Mimivirus in 2003 sent a shockwave through the virology community. The virus was 750 nm in size—gargantuan compared to typical viruses—and was termed a *girus. It contained the largest known viral genome, at a whopping 1.2 kbp of dsDNA, coding for 911 gene products.* It contained many genes not found before in other viruses, such as genes for **aminoacyl-tRNA synthetases** that are important components of cellular translation machinery and genes associated with metabolic pathways. In addition, four of its genes contained introns. *But Mimivirus still appeared to be absolutely dependent on its host cell for synthesis of its proteins.*

Following the deciphering of the Mimivirus genome sequence, efforts began to elucidate the replication cycle of the virus. Research teams led by Didier Raoult and Abraham Minsky conducted extensive ultrastructural studies to determine the replication cycle of Mimivirus. In their studies, Mimiviruses were "fed" and allowed to infect *Acanthamoeba polyphaga* at a cell-to-virus ratio of 1:10. At various times within the 24-hour post-infection period, the infected cells were collected and prepared for transmission electron microscopy. Transmission electron microscopy demonstrated that within 30 minutes the Mimiviruses entered amoebae by phagocytosis. Upon engulfment, the Mimiviruses underwent uncoating by fusing with the host cell phagosomes. During the fusion event, the viral capsids morphed into five-fold, star-shaped structures that acted like a "stargate," or portal, through which viral DNA was released into the cytoplasm of the host cell. Subsequently, the viral DNA was imported into the host nucleus where its first round of replication began. Like poxviruses, Mimiviruses possess their own transcriptional apparatus for viral mRNA synthesis.

By 3 hours post-infection, Mimivirus DNA exited the host nucleus to form cytoplasmic viral replication factories surrounded by mitochondria. By 5–8 hours post-infection, the viral factories increased 50% in size and viral proteins were detected. In addition to transmission electron microscopy, direct fluorescent staining was performed on the infected cells in order to study the viral factories. The factories appeared

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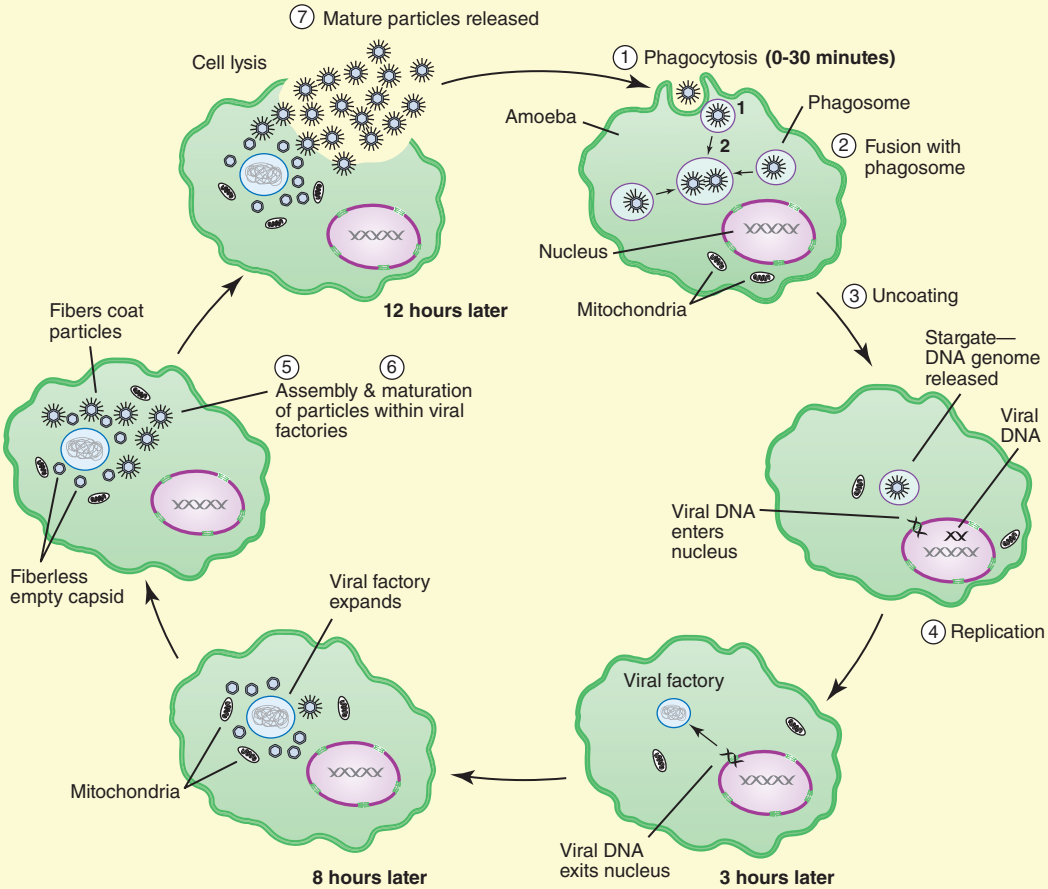
VIRUS FILE 3-3 Unraveling the Replication Cycle of Mimivirus (continued)



Information from Suzan-Monti, M., et al. 2007. "Ultrastructural characterization of the giant volcano-like virus factory of *Acanthamoeba polyphaga* Mimivirus." *PLoS ONE* 3:e328.

FIGURE 1 The Mimivirus replication cycle based on observations of infected amoeba cells at various times after infection.

to have three zones: an electron-dense inner replication center, an intermediate assembly zone, and a peripheral zone where particles matured and acquired fibrils. At 8 hours after infection, the viral factories contained empty, fiberless capsids that were partially assembled, as well as some icosahedral capsids undergoing DNA packaging through a transient "stargate," or aperture. By 10–12 hours post-infection, mature fibril-coated particles budded from the viral factories and were released through cell lysis (**FIGURE 1**). Overall, the takeover of the cellular machinery by Mimivirus was rapid and efficient. Many of the viral events in the replication cycle took place in a giant volcano-like viral factory.

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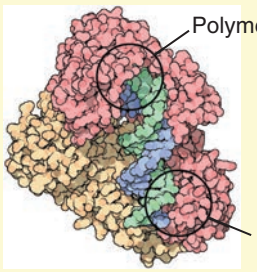
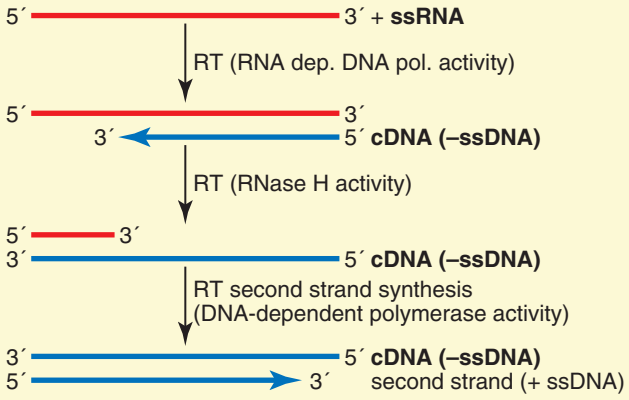


What is reverse transcriptase?
Reverse transcriptase (RT) has three distinct enzymatic activities:

1. RNA-dependent DNA polymerase
2. RNase H activity (cleaves/degrades RNA from RNA/DNA hybrids)
3. DNA-dependent DNA polymerase



Retroviruses and hepadnaviruses utilize RT in their life cycles.

Courtesy of David S. Goodsell, Scripps Research Institute. Reproduced from J. Ding, et al. 1998. "Structure and functional implications of the polymerase active site in a complex of HIV-1 RT with a double-stranded DNA template-primer and an antibody Fab fragment at 2.8 Å resolution." *J Mol Biol* 284:1095–1111.

FIGURE 1 Reverse transcriptase structure and function.

associates with cellular histones and is transcribed into separate viral mRNA transcripts and a full-length ssRNA pregenome.

The viral mRNAs are translated to yield the hepatitis B core antigens and the viral reverse transcriptase. The RNA pregenome associates with the viral reverse transcriptase and is packaged with the core proteins to form an **immature virus particle** in the cytoplasm of the cell. The viral reverse transcriptase synthesizes the ssDNA strand using the ssRNA intermediate as a template (see ***Refresher: Molecular Biology***, which is about reverse transcriptase functions). The pregenome is degraded by the RNase H activity of the reverse transcriptase enzyme, but it leaves a short sequence of RNA at its 5′ end that acts as a primer for DNA polymerase to synthesize a complementary +ssDNA strand in the **mature virus particle**.

Hepatitis B is one of a few known nonretroviral viruses that use reverse transcription as part of its replication process. Other viruses that utilize reverse transcriptase are retroviruses such as human T-cell leukemia virus (HTLV) and HIV, which possess a +ssRNA genome. The reverse transcription step of retroviruses is one of the first steps in viral replication, whereas for hepatitis B virus reverse transcription occurs during maturation (the later steps) in making new virus particles. In contrast to retroviruses, HBV does not possess **integrase** activity. Integrated parts of the hepatitis B genome, however, are found in the chromosomes of hepatocellular tumors from cancer patients. Retroviruses have integrase activity.

RNA Viruses

The genome replication of most RNA viruses occurs in the cytoplasm of the host. Presumably, this is because their replication is associated with RNA-dependent RNA polymerases that the host cell nucleus cannot provide.

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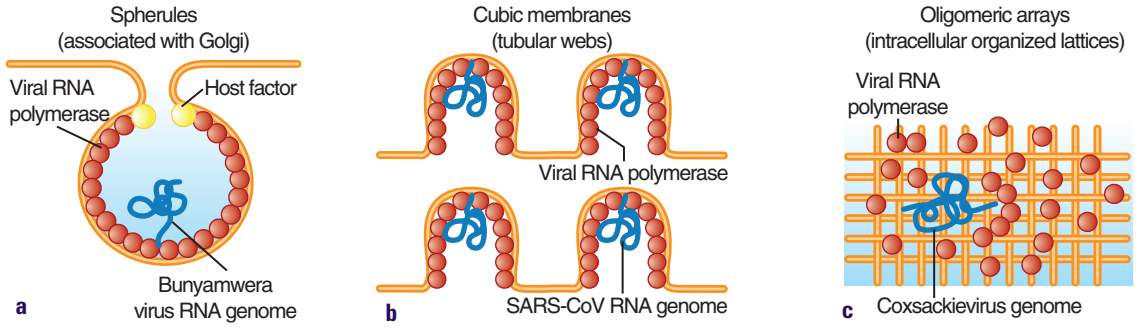
RNA viruses are unique because their genetic information is encoded in RNA. The genomes of RNA viruses are diverse (ss or ds, [+] or [–] sense, linear or segmented). The type of RNA genome determines whether the first step after uncoating will be translation, transcription, or RNA replication. The majority of viruses that replicate in the cytoplasm create **replication organelles** or membranous structures within the cell. There are three categories of replication organelles: single membrane **sphericules**, **cubic membranes**, and **oligomeric arrays** (**FIGURE 3-32**). The membranous structures form as necklike invaginations with a narrow opening to the cytosol of the cell to restrict the entry of undesirable materials, and they protect the viral genome from degradation as well as controlling the exit of the newly synthesized viral genomes. The structures occur within a variety of organelles, such as the mitochondria, endoplasmic reticulum, lysosomes, peroxisomes, chloroplasts (of plant cells), or Golgi apparatuses.

dsRNA Viruses The dsRNA viruses are faced with the challenge of host cells not producing RNA-dependent RNA polymerases to transcribe one of the viral RNA strands in the dsRNA into a +ssRNA that can be translated by the host cell machinery. To circumvent this molecular hurdle, dsRNA viruses carry their own RNA-dependent RNA polymerase within the virion particle. The viral RNA-dependent RNA polymerase can



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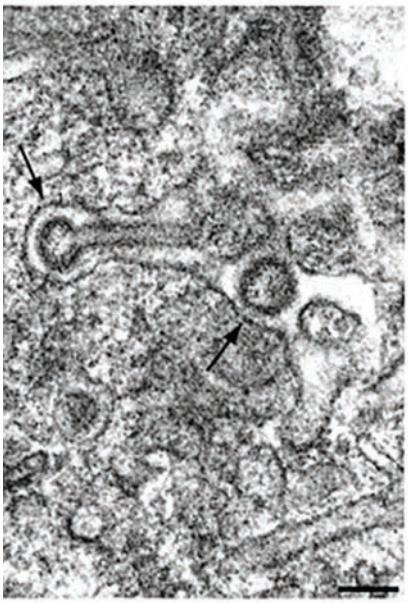

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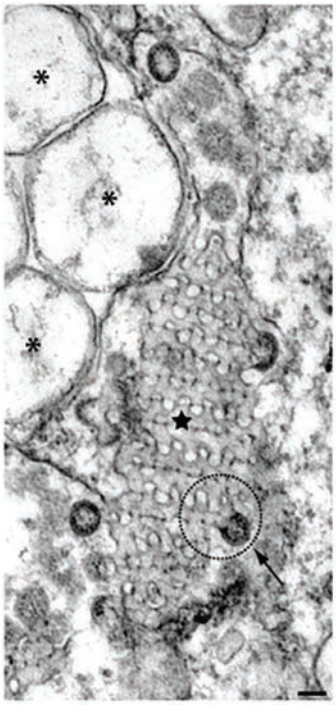

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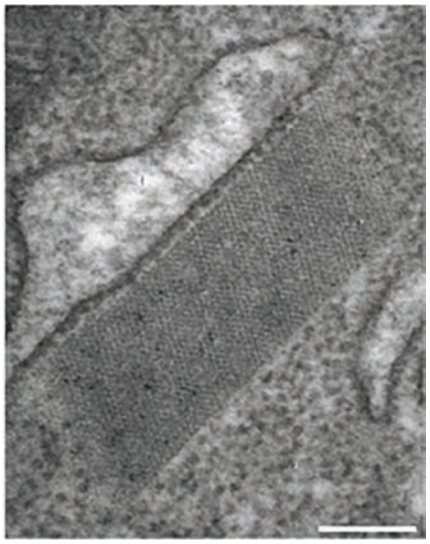

Reproduced from Kemball, C.C., Alirezaei, M., Flynn, C.T., Wood, M.R., Harkins, S., Kiosses, W.B., and Whitton, J.L. (2010) Coxsackievirus infection induces autophagy-like vesicles and megaphagosomes in pancreatic acinar cells in vivo. *J Virol* 84: 12110–12124. Used with permission.

FIGURE 3-32 Illustration of the three categories of viral replication organelles built by RNA viruses that replicate in the cytoplasm: **(a)** sphericules, **(b)** cubic membranes, **(c)** and oligomeric arrays. **(d)** Baby hamster kidney cells (BHK-21) infected with Bunyamwera virus. Bunyamwera virus is an enveloped –ssRNA virus. Arrows indicate the spherules that are associated with Golgi membranes. **(e)** Vero cells infected with SARS-CoV, an enveloped virus containing a +ssRNA genome. The replication organelles consist of cubic membranes (star) in contact with double-membrane vesicles (asterisks). The dashed circle surrounds a group of membranes connected with a budding virus. **(f)** Oligomeric lattice/arrays containing coxsackie virus components is adjacent to the rough endoplasmic reticulum inside of infected pancreatic acinar cells. Coxsackie viruses are naked and contain a +ssRNA genome.

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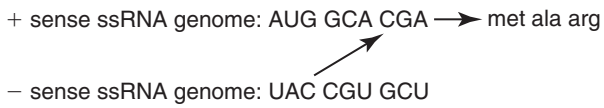

FIGURE 3-33 Differences between positive (+) and negative (–) sense ssRNA viral genomes. The (–) sense ssRNA is an antisense ssRNA that must be transcribed into a (+) sense ssRNA in order to code for a functional peptide or protein.

recognize dsRNA and transcribe the –ssRNA strand into a +ssRNA or viral mRNA that can be translated by the translation machinery within the cytoplasm of the host cell.

+ssRNA Viruses Viruses that contain +ssRNA genomes can be directly translated using the host cell protein synthesis machinery, because the +ssRNA acts like mRNA (**FIGURE 3-33**). All other types of RNA viruses (–ssRNA or dsRNA) must be transcribed into mRNA (+ssRNA) before translation can occur using the host cell machinery. Eucaryotic host cells do not contain RNA-dependent RNA polymerases that can transcribe the viral RNA genomes, and as a result these viruses must carry an RNA-dependent RNA polymerase that will synthesize the viral +ssRNA and –ssRNA intermediate to create viral genomes within the host cell. A simplified illustration of the replication cycle of an enveloped +ssRNA virus is shown in **FIGURE 3-34**.

–ssRNA Viruses All of the –ssRNA viruses encode their own RNA-dependent RNA polymerases that transcribe the –ssRNA genome into viral monocistronic +ssRNAs that can be recognized by the host cell translation machinery. The second function of the viral RNA-dependent RNA polymerase is to synthesize the viral/progeny genome using the +ssRNA as a template. Hence, the RNA-dependent RNA polymerase is sometimes referred to as having a **transcriptase** and **replicase** function.

+ssRNA Genomes Using a dsDNA Intermediate The *Retroviridae* family contains retroviruses that have been identified in virtually all organisms, including invertebrates. This suggests that these viruses have an evolutionarily successful design. Their biology is quite unique. The main focus on retroviruses has been on the avian (chicken) and human retroviruses: Rous sarcoma virus (RSV, discovered in 1911), HIV (discovered in 1983), and human T-cell leukemia viruses (HTLVs, discovered in 1981). Retrovirus infections cause a wide spectrum of diseases, including cancers, immune deficiencies, and neurological disorders. Most retroviral infections, however, occur without having any detectable, deleterious damage to the host.

The replication cycle of retroviruses includes the integration of the viral **complementary DNA** (**cDNA**) into the chromosomal DNA of the host cell. The result of this integration event is that the retroviral DNA is

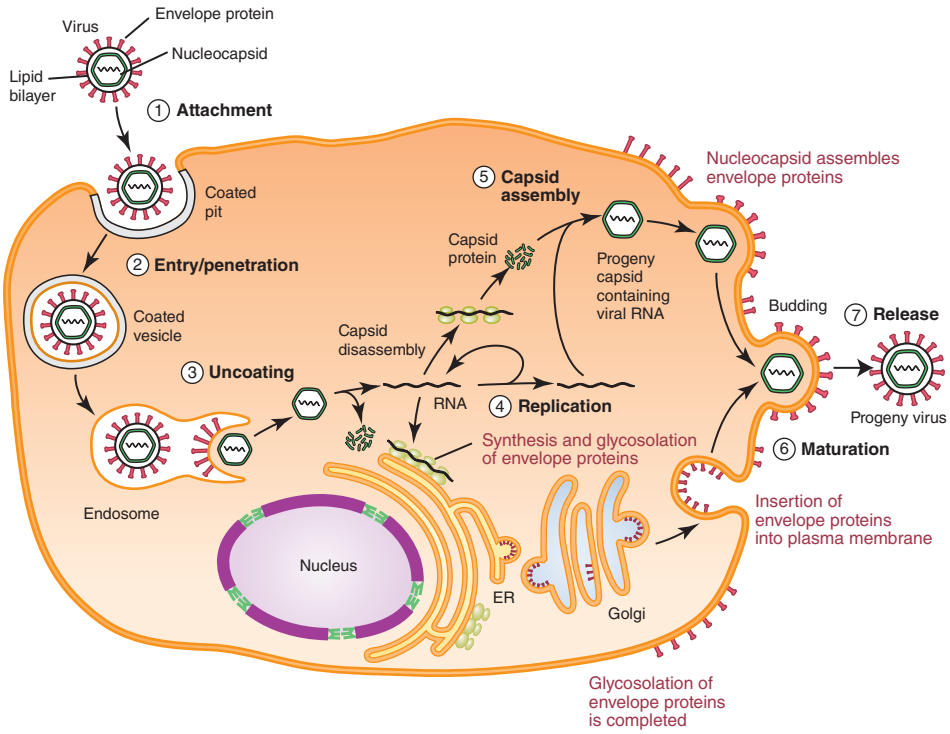

FIGURE 3-34 Example of a replication cycle of a +ssRNA-enveloped virus. All steps of the replication cycle take place in the cytoplasm. Like a typical eucaryotic mRNA, the viral +ssRNA genome is directly translated by the cellular ribosomal machinery in the cytoplasm. Viral glycoproteins are synthesized on and inserted into the rough endoplasmic reticulum (ER) membrane, where they are subsequently transported to the trans-Golgi network and then inserted into the plasma membrane. A viral polymerase synthesizes the viral genome. Newly synthesized capsid proteins bind to the replicated genome, forming a nucleocapsid that buds out of the plasma membrane to form the final enveloped virion.

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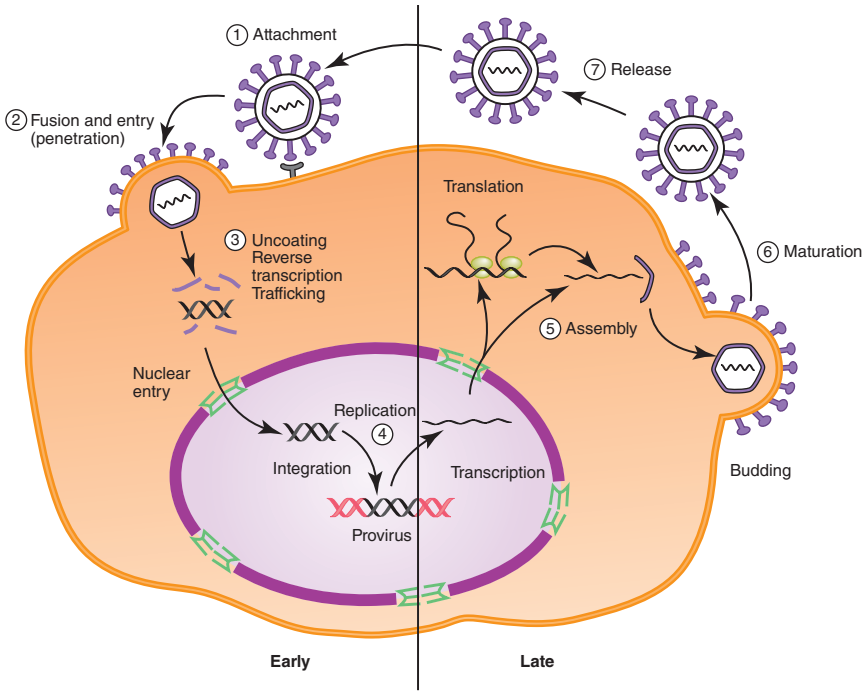

Information from an illustration by Kate Bishop, MRC National Institute for Medical Research, UK.

FIGURE 3-35 Basic replication cycle of a typical retrovirus. Retroviruses undergo a **latent** phase in which the virus begins the steps of a typical virus replication cycle: virus attaches to a host cell receptor(s) followed by uncoating of the virus particle in which the genome is translocated (also referred to as trafficking) to the nucleus where the genome is reverse transcribed into dsDNA that is integrated into the chromosome of the host cell. At this point, the retroviral DNA is replicated along with the host chromosome. This early stage is latent in that no infectious particles are made even though the retroviral DNA has integrated into the chromosomal DNA. After an "activation" event (or a productive phase in which viral particles are produced), the integrated retroviral DNA is transcribed. Viral mRNAs are exported to the cytoplasm where the cellular translational machinery produces the viral proteins. The capsid proteins and retroviral RNA are assembled/packaged into a nucleocapsid that buds from the plasma membrane, forming the final particle that undergoes a maturation step before the virus is fully infectious.

inherited from parent to offspring of the infected host if **germline cells** (sperm and egg) contain the integrated viral genome. These are termed **endogenous retroviruses** or **proviruses**, and their biologic properties and functions are still under investigation. Retroviruses that are not integrated into the germline cells of their hosts are called **exogenous retroviruses** (or external viruses).

The retrovirus genomes contain two copies of a +ssRNA molecule that is reverse transcribed into dsDNA by a viral RNA-dependent DNA polymerase (reverse transcriptase) to produce an RNA–DNA hybrid, which, in turn, is converted to dsDNA. The viral dsDNA is inserted into the host's chromosomal dsDNA. The integrated DNA (provirus) is subsequently transcribed by the host's DNA-dependent RNA polymerase II. The viral mRNA transcripts are spliced and exported into the cytoplasm of the cell and translated by the cellular protein synthesis machinery. Some full-length +ssRNA transcripts will be packaged into the new retrovirus particles (**FIGURE 3-35**).

Step 5: Assembly

Historically, research directed toward virus assembly mechanisms received less attention because there was more interest in the mechanisms of viral gene expression and replication. However, within the last 10 years considerable new knowledge regarding the unique structures formed during viral assembly have become available through advanced **live-cell imaging technology** (see **VIRUS FILE 3-4**).

Virus assembly is a key step in virus replication cycles. It involves the process by which the immature virus particle is formed. Despite the structural diversity of virus particles, the repertoire of assembly mechanisms is limited. All of the components of the virus must be assembled to create a stable structure. Conversely, the newly assembled virus must accomplish disassembly to start a new infectious replication cycle. The assembly event occurs when an appropriate concentration of virus proteins and viral genomes is reached and localized in the specific cellular compartments within the infected cell called **viral factories**, or **viroplasm**. These factories may serve as a way for viruses to hide from host cell antiviral defenses.

The viral factories recruit cell and viral components in a macrostructure in which viruses assemble and mature. The locations of viral factories differ according to the virus and have some influence on how the virus particle is released. Cell membranes and the cytoskeleton are involved in the biogenesis of scaffolds, and mitochondria are present in many factories. It is speculated that the mitochondria may supply energy and other essential

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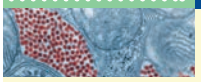
VIRUS FILE 3-4 Real-Time Virus Tracking in Live Cells



Viruses hijack cellular functions to optimize viral replication and virion production. Real-time, live-cell imaging techniques make it possible to follow the journey of individual virus particles inside of the host cell. The experiments entail imaging infected cells in culture. Viral components (e.g., capsid proteins or the viral envelope) are labeled with fluorescent dyes, and cellular structures are labeled with genetically encoded fluorescent proteins. **Fluorescence microscopes** are used to monitor individual viruses and cellular compartments, probing interactions between viruses and host cell machinery in real time.

This approach has improved our understanding of viral infection mechanisms and has shed light on fundamental questions in cell biology. Observing virus–host cell interactions in real time is helping researchers to dissect the steps of the infection process, such as the elucidation of endocytic pathways used for viral entry, uncoating mechanisms, viral assembly and egress, the reorganization of actin cytoskeleton (**FIGURE 1**), and intracellular trafficking of viral components during viral infection. Each step involves specific interactions between viral and cellular proteins (**FIGURE 2**).

Culture cells are simplified model systems. Efforts are under way to track virus particles in live tissues and animals to determine how viruses evade host cell defenses to reach target cells for infection. In 2006, Manchester and Stuhlmann led the first *in vivo* experiments tracking labeled viruses in mouse and chick embryos (see Lewis et al., 2006, in the References below).

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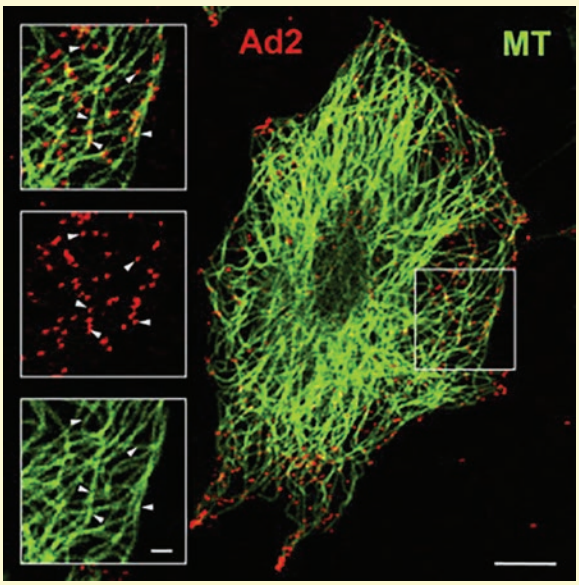

Information from "A Superhighway to Virus Infection", © 2006. Cell,124:4 , 741–754, with permission from Elsevier.

FIGURE 1 Confocal laser scanning microscopy showing dual-colored live-cell imaging of red-labeled adenovirus 2 particles interacting with microtubules (green) of HeLa cells. Scale bar: 10 µm. **Top inset**: Co-localization of adenoviruses (red) and microtubules (green). Arrowheads point to labeled adenoviruses or microtubules. **Middle inset**: Individually labeled adenoviruses in HeLa cells. **Bottom inset**: Individually labeled cellular microtubules of HeLa cells. Scale bar: 2 µm.

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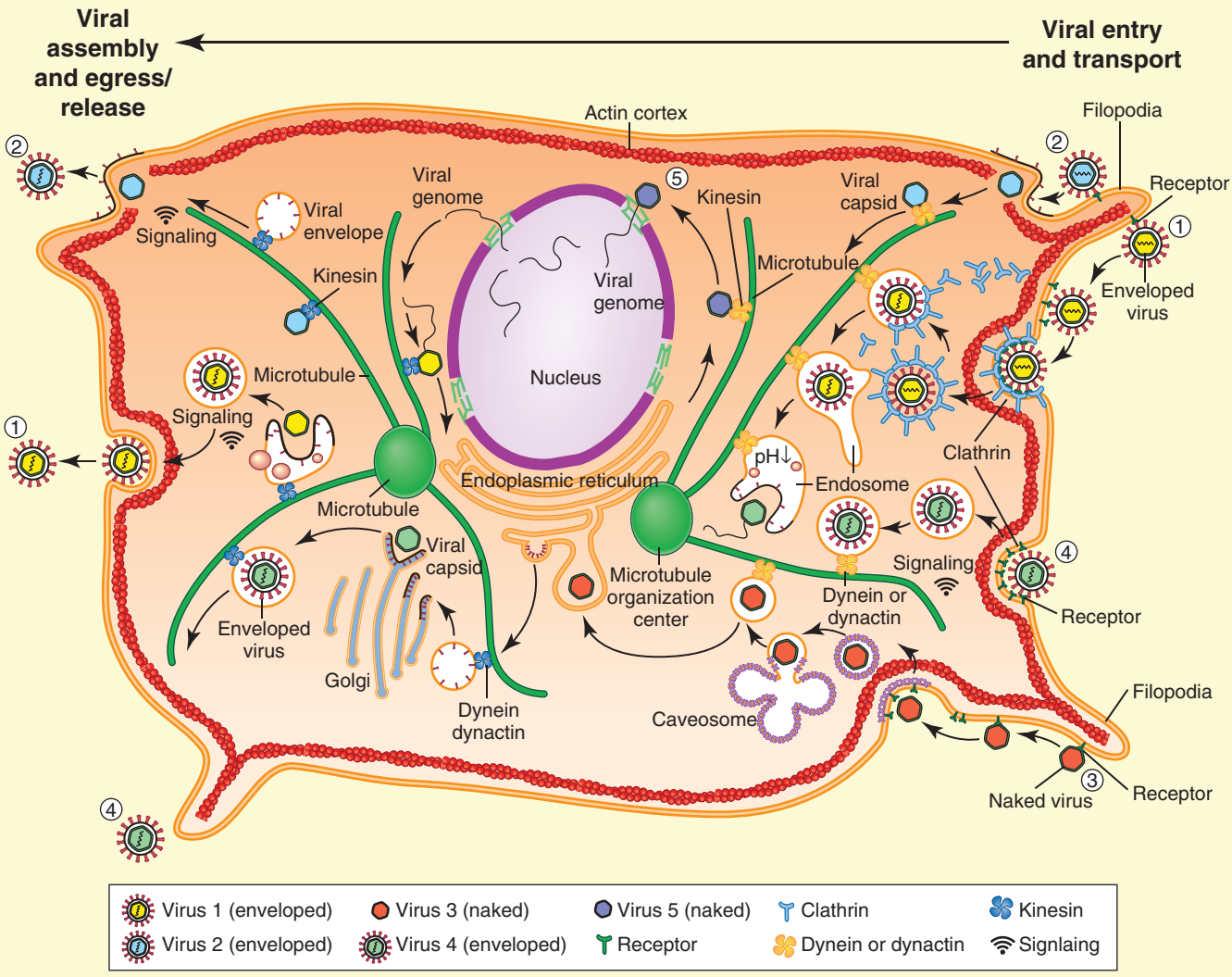

Information from Brandenburg, B., and Zhuang, X. 2007. "Virus trafficking—learning from single-virus tracking." *Nat Rev Microbiol* 5:197–208.

FIGURE 2 Multicolored fluorescent live-cell imaging techniques have provided information on the interactions between virus and cellular structures, further elucidating viral entry, transport, assembly, and egress mechanisms. This illustration represents virus–host interactions that occur during infection when actin filaments, microtubules, dynein/dynactin, kinesin, viral envelope proteins, viral capsid proteins, viral genomes, or signaling proteins are labeled with fluorescent or chemical tags/labels. It shows a host cell that has been infected with five different viruses in order to determine the diverse ways in which viruses enter and are transported within host cells, are assembled, and then released or allowed to egress. The viruses in the diagram are numbered 1–5. Each one is a different color (1, yellow; 2, blue; 3, red; 4, green; and 5, purple). Viruses surf the cell surface or move along the filopodia (1–3) and bind to specific receptors before entering a host cell. Viruses enter through direct fusion with the plasma membrane (2) or by (1) caveolin-dependent, (3) clathrin-independent, or (4) caveolin-independent endocytic pathways. After being internalized and transported through the actin cortex, vesicles that contain viruses are transported by microtubules driven by dynein or dynactin motors toward the microtubule-organizing center. Viruses may journey through endosomes (1, 4), caveosomes (3), or the endoplasmic reticulum (3) prior to release into the cytoplasm. Capsids (2) may be transported by microtubules driven by kinesin motors. Some viruses release their genomes into the cytoplasm (1); others release their genomes into the nucleus (5). Viral genomes are packaged into capsids and transported along the microtubules (1). Viral membrane proteins are translated at the endoplasmic reticulum (2) and transported along the microtubules to the Golgi apparatus, where capsids can bud into an envelope (3). Assembled virions inside of vesicles (4) or subviral particles (2) are transported on microtubules driven by kinesin motors and exit the cell by exocytosis (1) or by budding at the plasma membrane (2). During egress or release, actin tails may propel viruses towards neighboring cells (4).

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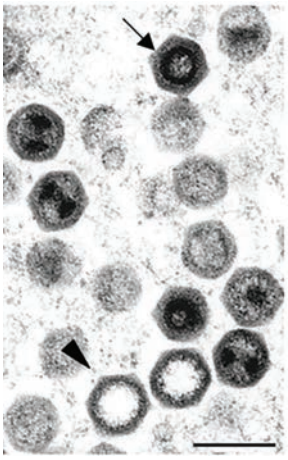
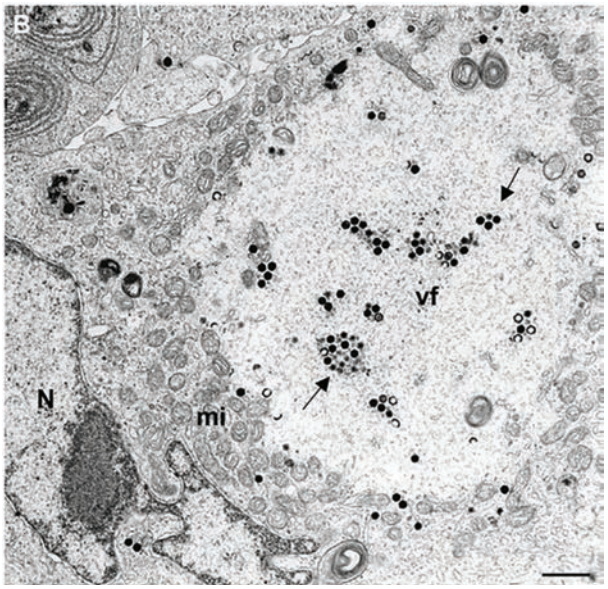
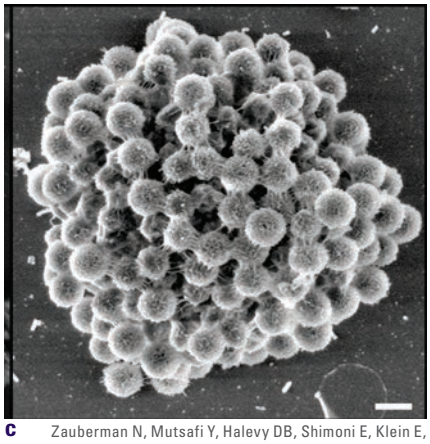


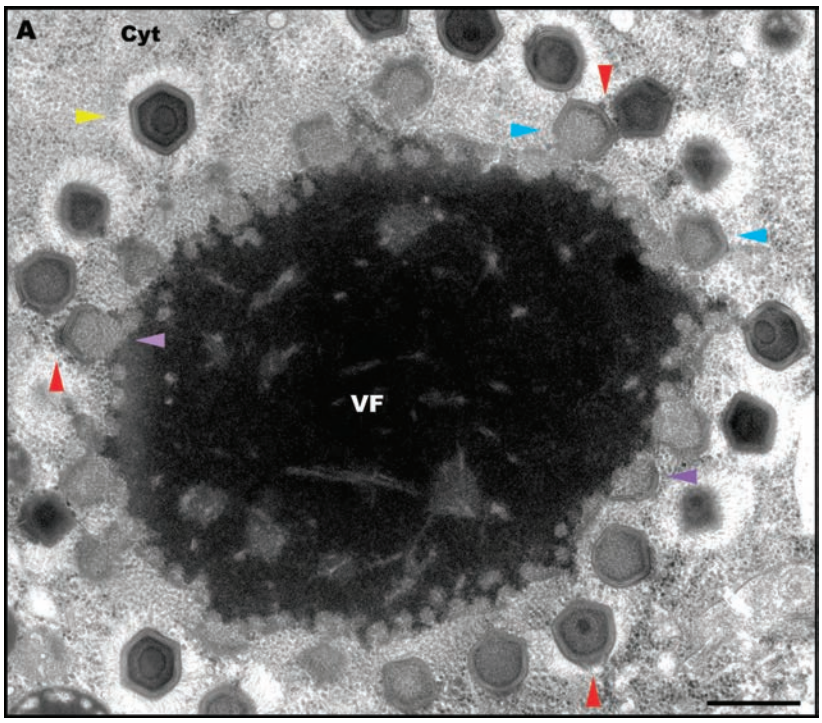
a Virus factories: associations of cell organelles for viral replication and morphogenesis, 2005. Novoa et al. Biology of the Cell 97:2, 147–172. With permission of Wiley.



b Virus factories: associations of cell organelles for viral replication and morphogenesis, 2005. Novoa et al. Biology of the Cell 97:2, 147–172. With permission of Wiley.



c Zauberman N, Mutsafi Y, Halevy DB, Shimoni E, Klein E, Xiao C, et al. 2008. Distinct DNA Exit and Packaging Portals in the Virus Acanthamoeba polyphaga mimivirus. PLoS Biol 6(5): e114. doi:10.1371/journal.pbio.0060114.



d Zauberman N, Mutsafi Y, Halevy DB, Shimoni E, Klein E, Xiao C, et al. 2008. Distinct DNA Exit and Packaging Portals in the Virus Acanthamoeba polyphaga mimivirus. PLoS Biol 6(5): e114. doi:10.1371/journal.pbio.0060114.

FIGURE 3-36 Examples of viral factories. **(a)** High-magnification electron micrograph showing ultrastructure of African swine fever virus (ASFV) factories. ASFV is a large enveloped dsDNA virus (approximately 200 nm in diameter). The arrow indicates full virus particles. The arrowhead indicates empty viral capsids. **(b)** Electron micrograph at lower magnification showing the ASFV factories. There are numerous mitochondria (mi) accumulating around the virus factory (vf). **(c)** Transmission electron micrograph of a thin section of a Mimivirus factory at various assembly stages. Scale bar: 500 nm. **(d)** Scanning electron micrograph of a Mimivirus factory containing mature virus particles isolated from infected amoeba cells. Scale bar: 500 nm.

factors for viral assembly. Some viruses build several mini-factories, whereas others create a single large factory (**FIGURE 3-36**).

Step 6: Maturation

The virus becomes infectious during the maturation step of the replication cycle. Viral or cellular proteases are often involved in the processes of maturation. One or more capsid or envelope proteins may undergo specific proteolytic cleavage within the particle. The cleavage event results in a subtle structural change of the virus particle, increasing its stability.

Virus-encoded proteases are attractive targets for antiviral therapies; for example, the protease inhibitors saquinavir mesylate (Invirase), ritonavir (Norvir), indinavir (Crixivan), and nelfinavir mesylate (Viracept) target the HIV-encoded protease by preventing the maturation of virions capable of infecting other cells (**FIGURE 3-37**).

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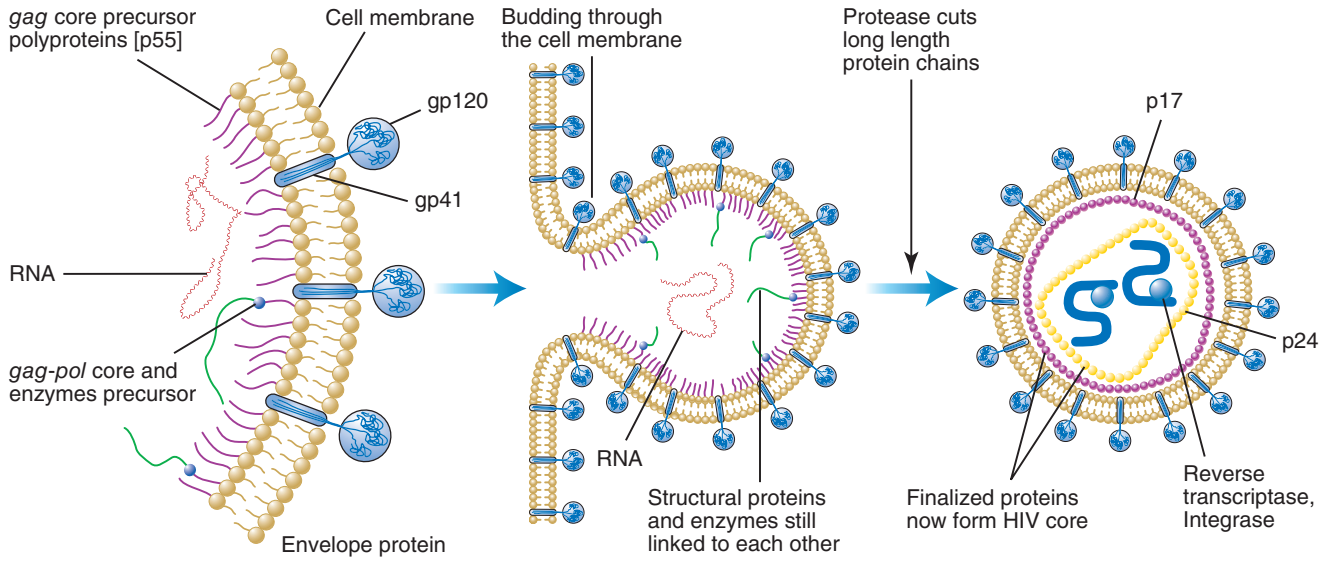


Information from Vella, S.1995. "Clinical experience with saquinavir." *AIDS9*(Suppl):S21–S25.

FIGURE 3-37 The structural proteins within the immature HIV virus particle must be cleaved by a viral protease inside of the particle in order for the virus to be infectious.

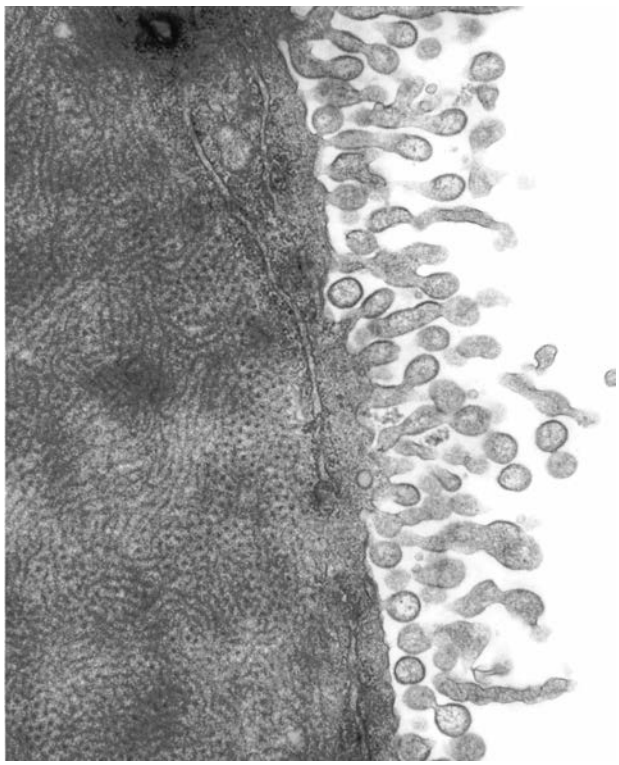
Step 7: Egress/Release

The egress (release) of virus particles poses an interesting dilemma. Viruses are designed to enter rather than leave cells. Newly formed viruses face the same barriers leaving the host cell that were encountered upon entry, such as host cell receptors for attachment and the actin meshwork located under the plasma membrane. Newly formed viruses egress the host cell upon **lysis**, escaping the cell as it disintegrates (**lytic viruses**) or by budding (**FIGURE 3-38**) through the plasma membrane of the cell. Viruses that egress by budding may or may not damage the cell. Other viruses bud from other membranes, such as the nuclear membrane, and exit from the cell via a secretory-like mechanism.

To overcome getting stuck on the outside of host cells during egress, some viruses produce an enzyme during their replication cycle to destroy the cellular receptors as the newly assembled infectious virions exit the cell. The influenza A viral protein, **neuraminidase**, cleaves the sialic acid receptors found on the outside of cells. As a result, the influenza A viruses do not aggregate at the cell surface.

Some viruses require the actin cytoskeleton to successfully exit and spread to uninfected host cells. Early evidence between virus egress and the actin cytoskeleton involved experiments in which mammary tumor cells infected with the retrovirus mouse mammary tumor virus (MMTV) were pretreated with **cytochalasin D**. Cytochalasin D is a cytodisruptive agent that changes the organization of actin within a cell. Cytochalasin D dramatically blocked MMTV budding and yield of virus particles.

Measles (a paramyxovirus), HSV, HIV, MMTV, and vaccinia virus (a poxvirus) have been shown to interact with actin filaments during replication and egress. Measles virus buds off the cellular plasma membrane at the ends of microvilli-like structures that contain internal actin filaments. Actin filaments interact with HSV-1 capsids docked on the nuclear membrane. HIV buds out of the plasma membrane by stimulating the actin cortex to fill microvilli underneath the virions as the virus exits the cell (**FIGURE 3-39A–C**). **Actin comet tails** present in lamellipodial extensions propel vaccinia virus through the plasma membrane of the host cell and are essential for viral spread to uninfected host cells (**FIGURE 3-39D**).



Courtesy of Shmuel Rozenblatt, Tel Aviv University, Israel.

FIGURE 3-38 Transmission electron micrograph of measles virus released by budding.

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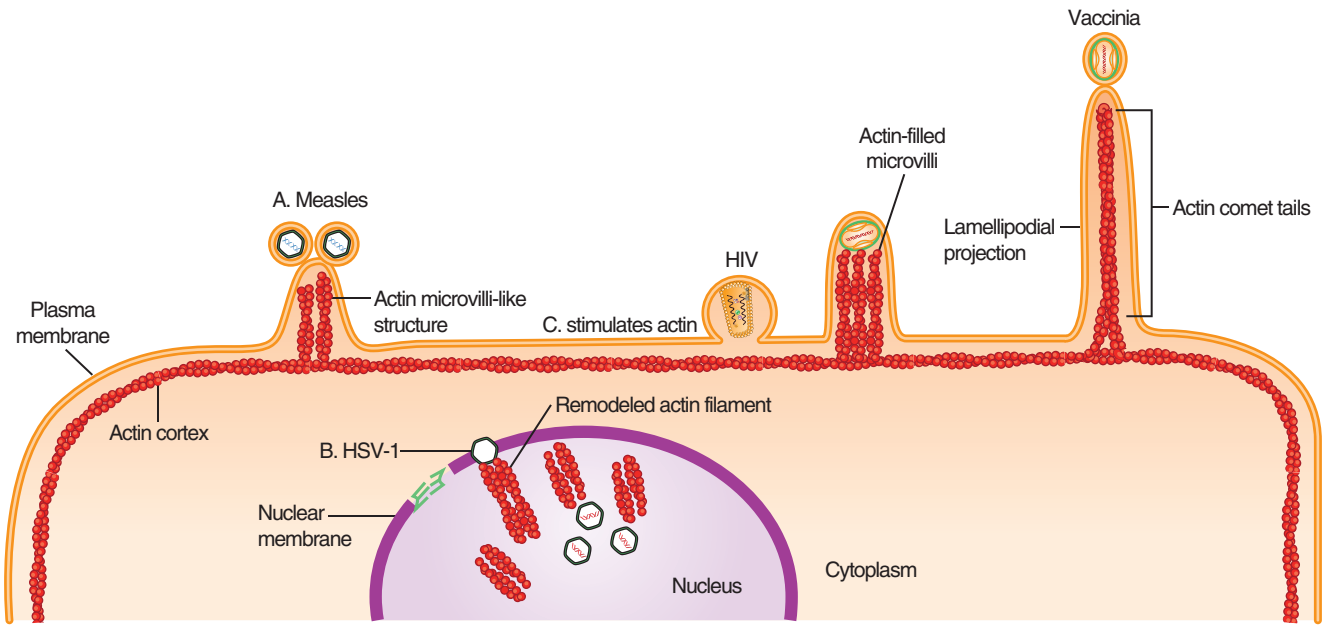

Information from Taylor, M. P., et al. 2011. "Subversion of the actin cytoskeleton during viral infection." *Nat Rev Microbiol* 9:427–439.

FIGURE 3-39 Using actin cytoskeleton during viral egress. **(a)** Measles virus budding at the ends of actin-filled microvilli. **(b)** HSV-1 interacts with actin filaments during assembly and egress at the nuclear membrane of the cell. **(c)** HIV stimulates the actin remodeling into microvilli-like projections to facilitate HIV egress at the plasma membrane of the host cell. **(d)** Vaccinia virus is propelled through the plasma membrane by the movement of actin comet tails inside of lamellipodial projections.

3.6 The Error-Prone RNA Polymerases: Genetic Diversity

Viruses replicate rapidly. During the process of replication, an error, or point mutation, may occur. The mutation rate of DNA viruses is usually similar to those of their cellular hosts because most of the polymerases used to replicate DNA genomes possess **proofreading ability**; for example, the mutation rate of herpesviruses (with proofreading ability) is one error in every 10^8 to 10^{11} bases. The genome sizes of herpesviruses range from 1.3×10^5 to 2.0×10^5 base pairs in length. As a result, herpesviruses potentially evolve very slowly because few mutations will be made, if any, during the infection cycle of the virus.

RNA viruses possess mutation rates as high as one error in 10^3 to 10^4 bases. The RNA-dependent RNA polymerases and RNA-dependent DNA polymerases (e.g., reverse transcriptases) used by the RNA viruses for genome replication do not possess proofreading ability. Hence, mutation of a virus population during each replication cycle of the virus occurs much more rapidly than in DNA viruses or cellular organisms. HIV and coronaviruses such a SARS-CoV are excellent examples of RNA viruses with high mutation rates. These viruses misincorporate a nucleotide into their genome once every 10^3 to 10^4 bases. For coronaviruses, this means that three mutations will occur during the replication of one viral genome. For HIV, this means one to two mutations in every genome copied. The high mutation rate probably limits the size of most RNA virus genomes to approximately 10^4 nucleotides.

Many mutations are lethal because the mutated virus is unable to replicate. Nonlethal mutations may give the mutated virus a selective advantage. Mutations have been associated with the development of antiviral drug resistance (such as the drug-resistant strains of HIV), changes in virulence (e.g., the 1997 Hong Kong avian influenza A was highly virulent), changes that allow the virus to evade the host's immune system, and changes in host range. A good example of a change in host range adaptation occurred in 1978. Canine parvovirus suddenly began killing large numbers of dogs globally in 1978. The parvovirus originally had infected only cats, foxes, raccoons, and minks. A small number of changes in the capsid genes adapted the virus for efficient spread among dogs.

3.7 Targets for Antiviral Therapies

Most researchers did not believe that *selective* antiviral drugs could be developed because of the fact that viruses are intracellular pathogens that are highly dependent upon host cell functions. Key to antiviral drug development is that the drug must *target a process essential for viral replication and it must be active against the virus without being "toxic" to the host organism*. It has been difficult to develop

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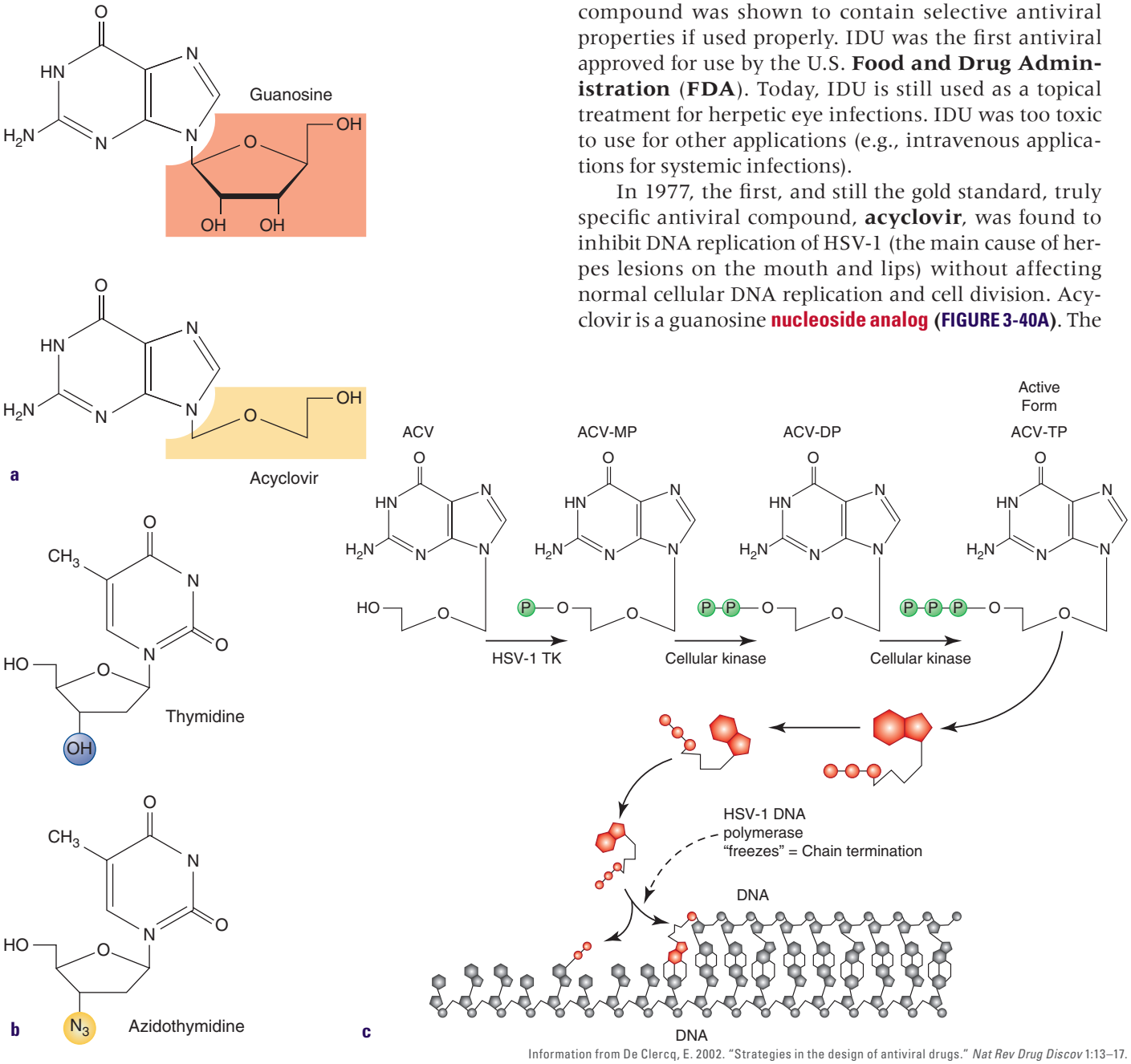
antivirals that have no toxic side effects for the host because viruses use some of the host cellular processes for replication. Hence, drugs cannot target those cellular processes because of toxicity problems for the host.

The era of antiviral drugs began in the 1950s when **5′ iododeoxyuridine** (now known as **idoxuridine**, or **IDU**), an analog of thymidine, was synthesized by pharmacologist William Prusoff. It was synthesized with the intention of being used as a chemotherapeutic drug to treat cancers. In 1961, E. C. Herrmann demonstrated that iododeoxyuridine inhibited HSV-1 and vaccinia virus. In 1962, a clinical trial led by Herbert E. Kaufman found that topical iododeoxyuridine cured herpes keratitis, a viral infection of the eye. This was a game-changer, because it was the first time that an antiviral compound was shown to contain selective antiviral properties if used properly. IDU was the first antiviral approved for use by the U.S. **Food and Drug Administration** (**FDA**). Today, IDU is still used as a topical treatment for herpetic eye infections. IDU was too toxic to use for other applications (e.g., intravenous applications for systemic infections).

In 1977, the first, and still the gold standard, truly specific antiviral compound, **acyclovir**, was found to inhibit DNA replication of HSV-1 (the main cause of herpes lesions on the mouth and lips) without affecting normal cellular DNA replication and cell division. Acyclovir is a guanosine **nucleoside analog** (**FIGURE 3-40A**). The



Information from De Clercq, E. 2002. "Strategies in the design of antiviral drugs." *Nat Rev Drug Discov* 1:13–17.

FIGURE 3-40 Mechanism of action of antivirals that are nucleoside analogs. **(a)** Chemical structures of acyclovir, which is a nucleoside analog of guanosine. **(b)** Chemical structure of azidothymidine, which is a nucleoside analog of thymidine. **(c)** Acyclovir is a guanosine nucleoside analog (it looks like a guanine nucleotide to the cellular DNA polymerase). Before acyclovir is active or is recognized by DNA polymerase it must be in the triphosphate form inside of a cell. The first phosphorylation step is performed by the herpes simplex virus (HSV) or varicella zoster virus (VZV, cause of chickenpox and shingles)–encoded **thymidine kinase (TK)**, and therefore only cells infected with HSV or VZV will be affected by the action of acyclovir. The second and third phosphorylation events are performed by cellular kinases. After acyclovir is active in a triphosphate form, the cellular DNA polymerase incorporates it into a growing chain of DNA opposite a deoxycytosine triphosphate nucleoside. Acyclovir triphosphate is missing the 3′ hydroxyl group that is needed to add the next nucleoside triphosphate onto the growing chain of DNA. The DNA polymerase "freezes" and DNA replication stops. Hence, acyclovir acts as a DNA replication **chain terminator**.

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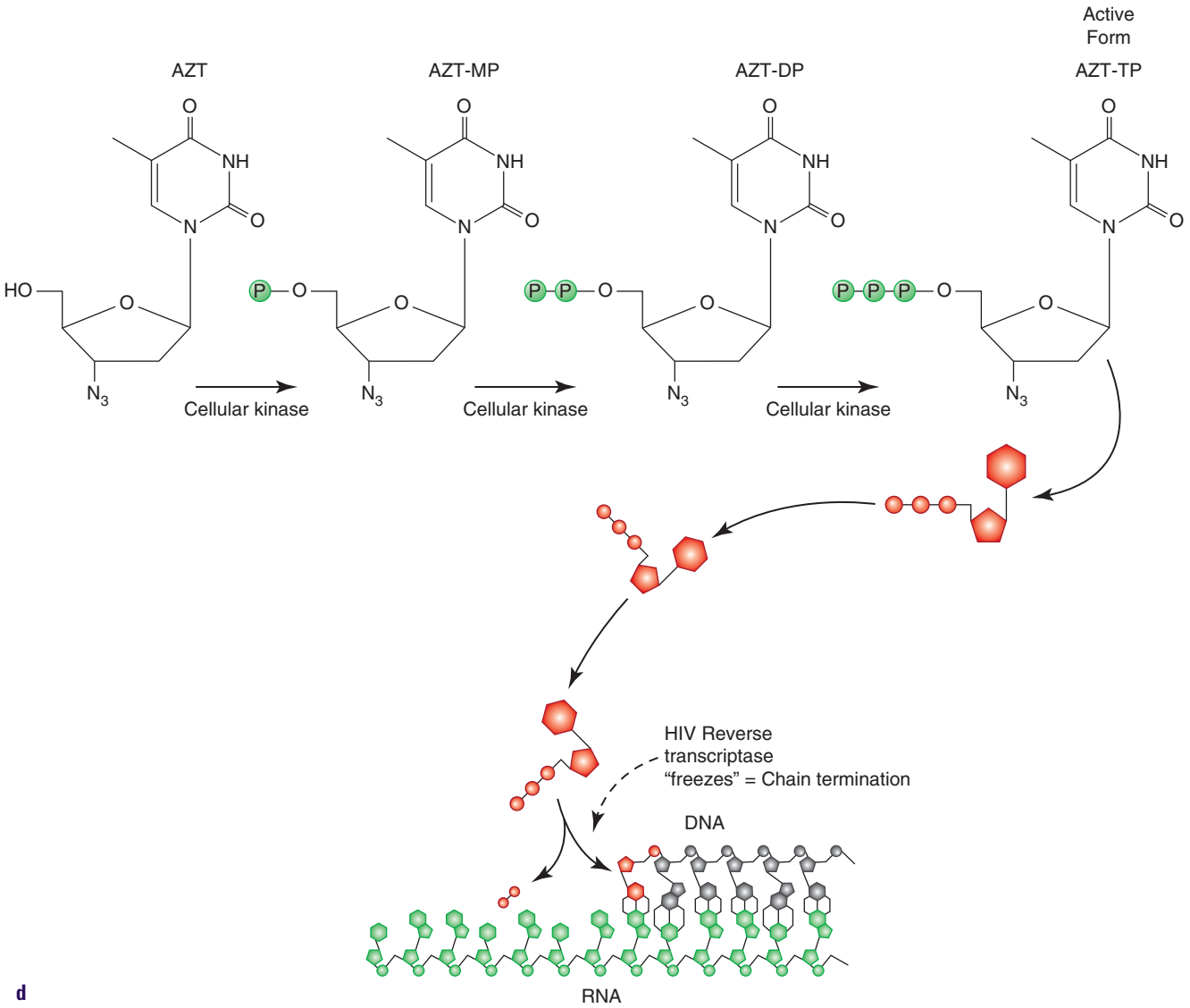

Information from De Clercq, E. 2002. "Strategies in the design of antiviral drugs." *Nat Rev Drug Discov* 1:13–17.

FIGURE 3-40 (d) Azidothymidine (AZT), a thymidine nucleoside analog, is phosphorylated in three steps by cellular kinases to the triphosphate form before it is active. Once active, the HIV reverse transcriptase incorporates AZT into a growing strand of DNA, but the AZT nucleoside triphosphate contains an **azido** (N_3) group instead of the 3′ hydroxyl group, causing the reverse transcriptase to freeze, leading to chain termination when AZT is incorporated into the growing DNA molecule.

antiviral research era started with the development of nucleoside analogs and really took hold in the 1980s when there was pressure to find a cure for HIV infection. The first antiviral drug approved by the FDA to treat HIV infection was **azidothymidine** (**AZT**), a thymidine nucleoside analog HIV reverse transcriptase inhibitor (**FIGURE 3-40B**). Nucleoside analogs cause DNA replication to terminate (**FIGURE 3-40C** illustrates the mechanism of chain termination). The scientific community began to grasp the concept that antivirals could be synthesized that had greater effect on virus replication cycles than on host cell functions. Any of the seven steps of the viral replication cycle could be targeted for antiviral intervention.

In 1990, just five antiviral drugs had been approved by the FDA to treat and sometimes cure viral infections. Today, there are about 50 licensed antivirals used to treat infections caused by herpes simplex viruses, cytomegaloviruses, hepatitis B and C viruses, HIV, and influenza A and B viruses (see **TABLES 3-4**, **3-5**, and **3-6**). The vast majority of antivirals inhibit virus-encoded enzymes such as DNA polymerases, integrases, proteases, and neuraminidase. In 2015, **daclatasvir** (Daklinza) was approved by the FDA to treat patients with chronic hepatitis C (genotype 3) virus infections. Daclatasvir is an inhibitor of the hepatitis C virus NS5A replication complex. It blocks two distinct stages of the hepatitis C virus replication cycle: viral RNA synthesis and virion assembly. Other antivirals target specific cellular proteins involved in virus–host interactions, such as **maraviroc**, which antagonizes the HIV chemokine coreceptor CCR5, and inhibitors of cyclophilins are in development to treat

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Table 3-4 FDA-Approved Antiviral Drugs Used to Treat HIV Infection

Generic Drug Name	Target
Abacavir sulfate (ABC), didanosine (ddI), emtricitabine (FTC), lamivudine (3TC), stavudine (d4T), tenofovir disoproxil fumarate (TDF), zidovudine (AZT), nevirapine (NVP), delavirdine (DLV), efavirenz (EFV), rilpivirine, etravirine	HIV reverse transcriptase inhibitor
Atazanavir sulfate (ATV), fosamprenavir calcium (FOS-APV), saquinavir mesylate (SQV), indinavir (IDV), darunavir, nelfinavir mesylate (NFV), ritonavir (RTV), tipranavir (TPV), lopinavir	HIV protease inhibitor
Enfuvirtide (T-20)	HIV gp41 fusion inhibitor
Maraviroc	CCR5 coreceptor antagonist
Raltegravir, dolutegravir	HIV integrase strand transfer inhibitor
1. Efavirenz + emtricitabine + tenofovir disoproxil fumarate (Atripla) 2. Emtricitabine + rilpivirine + tenofovir disoproxil fumarate (Complera) 3. Elvitegravir + cobicistat + emtricitabine + tenofovir disoproxil fumarate (Stribild) 4. Tenofovir + emtricitabine (Truvada)	Multiclass combination drugs

Table 3-5 FDA-Approved Antiviral Drugs Used to Treat Herpesvirus Infections

Herpesvirus	Generic Drug Name	Target
Herpes simplex viruses 1 and 2 (cutaneous disease; e.g., oral or genital herpes)	Acyclovir, valacyclovir, peniclovir	Viral DNA polymerase
Varicella zoster (chickenpox or shingles)	Acyclovir, valacyclovir	Viral DNA polymerase
Herpes B virus	Acyclovir, valacyclovir, ganciclovir	Viral DNA polymerase
Cytomegalovirus (disease in transplant recipients; antiviral prophylaxis for prevention of CMV in transplant patients; CMV retinitis in HIV patients)	Ganciclovir, valganciclovir, foscarnet, cidofovir, valacyclovir, fomivirsen	Viral DNA polymerase, antisense inhibition of CMV gene expression

Table 3-6 FDA-Approved Antiviral Drugs Used to Treat Other Viral Infections

Virus	Generic Drug	Target
Influenza A virus	Amantadine,* rimantadine*	Viral M2 ion channel function
Influenza A virus	Oseltamivir, zanamivir, peramivir	Viral neuraminidase
Hepatitis B virus (chronic infection)	Interferons α-2a and α-2b	Host innate defense
Hepatitis B virus (chronic infection)	Adefovir, emtricitabine, entecavir, lamivudine, telbivudine, tenofovir	Hepatitis B virus replication
Hepatitis C virus (chronic infections)	Interferons α-2a and α-2b or pegylated interferon-α-2a or pegylated interferon α-2b	Host innate defense
Hepatitis C virus	Boceprevir, Telaprevir, Simeprevir	Viral NS3/4A protease
Hepatitis C virus	Sofosbuvir	Viral NS5B (viral RNA polymerase)
Broad spectrum of viruses: Hepatitis C virus, respiratory syncytial virus, herpes simplex virus, SARS-CoV, Nipah virus, La Crosse encephalitis virus, hantavirus, Bolivian hemorrhagic fever, Lassa fever virus, Crimean-Congo hemorrhagic fever virus	Ribavirin	Viral replication, mRNA mutagen

*No longer prescribed in the United States.

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hepatitis C virus infections. **Cyclophilins** are a group of proteins with peptidyl-prolyl isomerase activity involved in a variety of functions related to cell metabolism and energy homeostasis.

Birinapant, a small synthetic molecule used as a cancer drug, is currently in clinical trials to treat chronic hepatitis B virus infection. In Australian preclinical trials, birinapant in combination with the antiviral entecavir was 100% effective in clearing hepatitis B virus infections in animal models. **Entecavir** is a nucleoside analog used to treat hepatitis B infections. It blocks replication of the hepatitis B virus genome. Cancer cells and cells chronically infected with hepatitis B virus evade **apoptosis** (programmed cell death), causing the deregulation of a family of **inhibitor of apoptosis proteins** (**IAPs**). IAPs are overexpressed by many types of cancer cells that suppress apoptosis by binding and inhibiting active **caspases** that induce apoptosis through signaling pathways in normal or uninfected cells. Birinapant inhibits IAPs, restoring and inducing apoptosis signaling pathways in cancer cells. Theoretically, the birinapant restores apoptosis in hepatitis B chronically infected cells, allowing entecavir to inhibit replication of the hepatitis B virus genome, stopping it in its tracks. Human phase I and II trials involving the drug combination of birinapant and entecavir to treat patients with chronic hepatitis B began in Australia in December 2014.

The unprecedented Ebola epidemic in West Africa prompted the need for therapeutic agents. At the time of the epidemic, apart from supportive care, no licensed vaccine to prevent Ebola virus infection nor any specific antiviral for the treatment of Ebola virus disease was available. Several promising therapeutics had been identified, such as ZMapp, which is a monoclonal antibody–based therapeutic that targets the surface glycoprotein of Ebola virus.

Scientists at pharmaceutical companies are now using their knowledge of viral replication cycles and **rational drug design** computational software such as **computer-aided drug design (CADD)** and **computer-aided molecular modeling** (**CAMM**) to drive the development of antivirals. Organic chemists chemically synthesize and "tweak" the **pharmacophore** of known antiviral compounds to make them more effective or potent in their inhibition of the target molecule (e.g., viral protease or polymerase) that ultimately leads to inhibition of viral replication. The term *pharmacophore* is derived from *phoros*, the essential features responsible for a drug's (*pharmacon*) biological activity. Pharmacophore approaches are associated with the discovery and development of new drugs.

It has become clear that it is not sufficient to have one antiviral drug to control each type of viral infection, because viruses continue to genetically diversify, resulting in the emergence of drug-resistant virus variants and chronic infections requiring treatment for years. Another area of research is in the prophylactic use of antiviral therapy to *prevent* viral infections in immunocompromised individuals (e.g., transplant recipients and individuals with HIV/AIDS). We are now in an era where the development of new classes of antiviral drugs is being directed not just at the steps of the viral replication cycle but also at host cell factors involved in virus replication, the host immune response, and virus–host cell interactions.

Sources of Novel Antivirals

The two main approaches to antiviral drug development are rational drug design using synthetic chemistry and conventional methods that involve random screening of compounds in cell culture. Automated equipment can be used to perform **high-throughput screening** of hundreds or thousands of compounds from natural sources in a reasonable time period. Natural sources of bioactive compounds may be plants, microbes, or marine organisms. The term **bioprospecting** has been adopted to describe searches for new bioactive compounds in natural sources with potential for commercial use.

Cragg and Newman surveyed new drugs developed for any infectious disease, cancer, or other health conditions over the period of January 1981 through December 2010. Of the 1,073 drugs that were surveyed, more than two-thirds of the active agents had some relationship to natural sources, and only 30% were of purely synthetic origin. Classic methods used to develop therapeutic drugs are slow and expensive. It can take up to 15 years and up to $800 million to develop a new drug.

Interest is growing in the use of **reverse pharmacology** as a way to screen natural products for bioactive compounds. The reverse pharmacology approach involves researching the history of traditional medicines to treat diseases used in different cultures. Herbal medicines were used based on trial and error. Those medicines with positive results were kept, and the "therapeutic wisdom" was passed on to subsequent generations through an oral tradition (**VIRUS FILE 3-5**). In theory, investigating traditional treatments for bioactive compounds *should save time and expenses in drug development costs as well as increase the chances that a remedy will be effective and safe*.

Data mining is in progress to retrieve knowledge from ancient texts. For example, for thousands of years traditional Chinese medicine has studied the diagnosis and treatment of human disease. It has become a significant complementary resource of information for modern biomedical sciences. Traditional Chinese medicine literature obtained from various historical periods as well as from modern clinical studies has recently been transformed into digital data in the form of databases and text documents, providing an effective platform for sharing and retrieval by data mining.

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VIRUS FILE 3-5 Antiviral Drug Discovery Through Reverse Pharmacology

Bioprospecting has been going on since the dawn of civilization when prehistoric people from different cultures gathered plants for use as medicines. A local knowledge base arose primarily from trial and error. The empirical knowledge was passed down orally or through repeated practice. Medicinal plant knowledge in many traditional cultures is quickly eroding, because many of their young people are being influenced by modernization and the accessibility of Western medicine. As a result, local medicinal practices are being abandoned. Healers are elderly and dying with their knowledge left unrecorded. **Ethnobotanists** have recognized the need to document and describe the use of medicinal plants by various peoples. Comparative studies of medicinal plants selected by humans across cultures, regions, and hemispheres are finding significant similarities and some differences in the patterns of selection and avoidance of different plants.

Despite the current stringent and demanding regulatory process, a number of FDA-approved drugs have been withdrawn from the market due to safety concerns. Drug discovery is at a crossroads in which the use of traditional medicine–inspired reverse pharmacology will save time and expense in drug development. Research is under way to retrieve knowledge from ancient texts and reports from missionaries to extract information on plant species used to treat disease. Scientists are interviewing and studying healers in order to document and publicize traditional medical knowledge (**FIGURE 1**). The **Tanga project** is an example of collaboration between traditional healers, physicians, and medical staff to manage HIV/AIDS in Tanzania. The Tanga AIDS Working Group (TAWG) used indigenous knowledge in an attempt to alleviate suffering from HIV/AIDS. Over 4,000 AIDS patients were treated by TAWG with herbs prescribed by local healers. The greatest success was achieved in reducing opportunistic diseases that accompany HIV infection.

The few antiviral drugs that are available specifically treat about a handful of viral infections (e.g., influenza A, herpesvirus, HIV, and hepatitis B and C). The majority of patients who experience viral infections are given supportive care to ease symptoms. There are over 220 known human viruses, and it has been

Photos a, b, c courtesy of Shawn McAfee, Moraine Park Technical College.

FIGURE 1 **(a)** Researcher Sitha Thor working with his mother, herbalist Seng Vang Thor, to document uses of Hmong herbal medicines brought from Laos. The herbs are now grown in a backyard garden in Appleton, Wisconsin. **(b)** Sitha Thor and herbalist Grandma Nao Pao Thao in a backyard garden located in Fresno, California. **(c)** Sitha Thor screening Hmong herbs for inhibition against several different viruses grown in cell cultures in the laboratory.

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VIRUS FILE 3-5 Antiviral Drug Discovery Through Reverse Pharmacology (continued)

predicted that about 320,000 human viruses may exist. In recent years, the threat of a viral epidemic or even a pandemic has become more of a reality than ever before. This is why there is an urgent need for new pharmaceuticals or neutraceuticals to treat viral infections. Natural product drug discovery based on reverse pharmacology may expedite antiviral drug discovery.

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Summary

All viruses rely, to varying degrees, on the metabolic processes of their hosts to replicate themselves. Their hosts challenge them in several ways. The ability of the virus to replicate inside of a host cell depends on whether the host cell contains the appropriate receptors for entry and the proteins needed to carry out viral replication, transcription, and translation. This chapter provides a review of eucaryotic molecular biology, discusses the hurdles that viruses must face as they hijack cells to replicate, presents the seven key steps in the replication cycle of a virus, and explores the targets and strategies used to develop antiviral therapies.

It is intended that as a consequence of your coursework, you will begin to understand viruses from a molecular biology perspective. In other words, you have been provided with insights to the molecular needs of viruses and with a few examples of strategies that viruses use to overcome cellular limitations. It should be noted, though, that much of what we know today about genes, replication, DNA repair, transcription and control, splicing and processing of RNA, translation of mRNA, and protein modifications have relied extensively on viruses as critical research tools. *Viruses have been our "eyes" into cells. The intimate association between a virus and its host has allowed us to understand how cells function*.

With the development of animal cell culture techniques, scientists were able to study virus replication through one-step growth experiments. The knowledge we have today is very detailed and continues to progress so rapidly that it is impossible to cover the viral replication strategies used by every family of viruses in a single chapter. Real-time live-cell imaging techniques have unlocked some of the secrets of the intricate details of virus entry, cytoplasmic transport, assembly, and egress of new virions. Replication strategies are carried out based on the nature of the viral genome type: DNA or RNA, single stranded or double stranded, segmented or nonsegmented.

Viruses are masters of mutation. Mutations can be lethal or nonlethal. Nonlethal mutations may give rise to mutants that may increase their survivorship; for example, mutations may increase infectivity, resulting in viral drug resistance, further antagonizing the host immune system, or broadening the host range of the virus.

Scientists are applying the knowledge obtained through molecular biology toward the discovery and development of antiviral drugs. Antivirals target viruses specifically to be effective in eliminating the virus without causing harm to its host. We are in an era in which new classes of antiviral drugs are in development that target host cell factors involved in replication, the host immune response, and virus–host cell interactions. Reverse pharmacology is emerging in the discovery of antiviral compounds from medicinal plants. The threat of antiviral drug resistance, new and reemerging viruses, and the threat of viral pandemics expedite the need for the development of more antiviral therapies.

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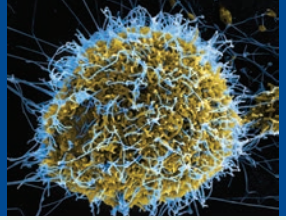

CASE STUDY 1: QUESTIONS

These questions relate to the Case Study presented at the beginning of the chapter. To find the answers, some questions may require researching the primary literature or using the resources provided.

1. Ebola virus can be detected in virtually all organs and many cell types. What are **dendritic cell-specific ICAM-3 grabbing integrin** (**DC-SIGN**) **lectins**, and what Ebola virus protein interacts with DC-SIGN during infection? During what step in the virus replication cycle does this virus–host interaction occur?
2. What is the function of **T cell immunoglobulin and mucin domain 1** (**TIM-1**), and what Ebola virus protein interacts with it? What role does it play in the replication cycle of Ebola virus?
3. Define **macropinocytosis**. Create a list of viruses besides Ebola virus that enter host cells by macropinocytosis.
4. What are the functions of **Niemann-Pick-C1** (**NPC1**), **cathepsin B**, and **cathepsin L** in cells? How do these proteins interact with Ebola viruses within an infected host cell?
5. After Ebola virus is inside of the cytoplasm of its host cell, what viral proteins suppress the cell's **innate immunity**? What innate responses are suppressed? How does this activity cause pathogenesis within the host?
6. The Ebola virus glycoprotein has two forms. How are the two forms produced, and how does each form function during a viral infection?
7. List the host cell machinery that Ebola virus hijacks during infection. Describe the viral mRNAs (e.g., are they capped? polyadenylated?). How does the phosphorylation of Ebola virus VP30 regulate transcription and replication processes within the infected cell?
8. Which Ebola virus proteins contribute to viral genome replication and transcription?
9. Draw a diagram showing the replication cycle of Ebola virus. Label the parts of the cell along with the viral and host protein interactions involved. Be sure to identify the Ebola and host proteins interacting during the replication cycle.
10. List and describe the mechanisms of antivirals in development that target different steps of the Ebola virus replication cycle.
11. Discuss why convalescent blood from an Ebola survivor is likely to improve the outcome of a patient suffering from EVD. What proteins in whole blood would be responsible for this action, and at what step in the virus replication cycle do the immune proteins inhibit Ebola viruses?
12. Perform research to create a list of antivirals that were approved by the FDA for *experimental use* to treat EVD during the epidemic in West Africa. What were the mechanisms of action for the experimentally approved drugs?

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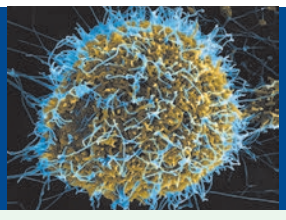

CASE STUDY 2: A RABIES VIRUS WITH AN ABORTIVE REPLICATION CYCLE?

In February 2009, a 17-year-old Texas teenager arrived at a hospital emergency room (ER) with symptoms of severe headache, neck pain, dizziness, **photophobia**, nausea and vomiting, and tingling of her face and forearms. The headaches had started 2 weeks prior to the ER visit. A **lumbar puncture** was performed, which revealed a very high white blood cell count, suggesting that the teen might be fighting a bacterial infection. She was treated intravenously with the broad-spectrum antibiotic ceftriaxone. No bacteria were cultured from her cerebral spinal fluid, so the antibiotic was discontinued.

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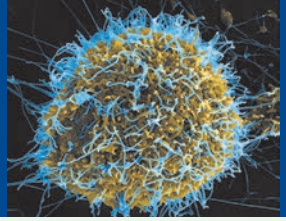

CASE STUDY 2: A RABIES VIRUS WITH AN ABORTIVE REPLICATION CYCLE? (continued)

After a 3-day hospital stay, she was released because her symptoms had resolved. About a week later, she returned to the hospital with the same symptoms. She also had a rash on her arms and back. **Magnetic resonance imaging** (**MRI**) was performed on her head. The MRI showed enlarged lateral ventricles inside her brain. The size was abnormal for her age, and she was given the diagnosis of **encephalitis**. She was hospitalized and treated with ceftriaxone and antibiotics used to treat tuberculosis.

Four days later, she continued to weaken. She became agitated and "combative." At this time, clinicians did an extensive workup, collecting the patient's history and creating a list of possible etiologies to explain the encephalitis/aseptic meningitis. A breakthrough came when the teenager mentioned that 2 weeks before her headaches began she had been hiking and spelunking (2 months prior, December 2008) and had brushed up against some bats, but didn't recall being bitten. Serum, saliva, and cerebral spinal fluid were collected from the patient. A **nuchal skin biopsy** was performed, and the clinical specimens were sent to the CDC for laboratory testing for rabies antibodies, rabies antigens, and/or the RNA genome of rabies virus.

The next day, no rabies RNA or virus antigens were detected. However, four serum samples tested positive for antirabies antibodies by direct fluorescent antibody tests. Antirabies antibodies were also found in the cerebral spinal fluid. Four days later, the teenager was given one dose of rabies vaccine and 1,500 international units (IU) of human rabies immunoglobulin. Eight days later, her symptoms resolved, and she was released from the hospital. Another week passed and she returned to the ER with recurring headache but left before a lumbar puncture was performed. A few more days passed. Again, she had a recurring headache. A lumbar puncture was performed. Her headache resolved, and she has not been hospitalized since. Only her boyfriend met the criteria of requiring a series of rabies vaccinations.

1. Create a hypothesis as to why this teenager survived a rabies virus infection.
2. What do the CDC researchers speculate as to why this patient survived rabies? (*Hint*: See Holzmann-Pazgal et al. [2010] in the References below.)
3. Assuming the teenager was not bitten by a rabid bat, what could be another plausible explanation for how the teenager contracted a rabies virus infection?
4. Rabies virus is a member of the *Rhabdoviridae* family. What are the molecular characteristics of this family of viruses (e.g., virion characteristics, replication cycle)?
5. How many genes does the rabies virus genome contain, and what are their function(s)?
6. What types of cells do rabies viruses replicate in? (*Hint*: See Table 3-2.)
7. Rabies viruses bind to what host receptor(s)?
8. Who should receive a rabies vaccination? (*Hint*: See http://www.cdc.gov/rabies/.)
9. Rabies virus kills 50,000 people globally a year. World Rabies Day is held September 28. Create a poster about rabies prevention for a World Rabies Day event.
10. A new strain of rabies virus was isolated from a rabid fox in New Mexico after it bit a 78-year-old woman. Perform research and summarize the evidence for claims that it is a new rabies virus variant.
11. Rabies was thought to be a 100% fatal disease in humans. New research has uncovered evidence that a small number of Peruvians possess natural immunity toward rabies viruses transmitted by bats in a remote region of the Amazon. Provide a rationale as to why blood samples drawn from this unvaccinated isolated population possess antibodies toward the rabies virus. Address both the human host and the rabies virus strain from bats captured in the remote Amazon forests in your answer.

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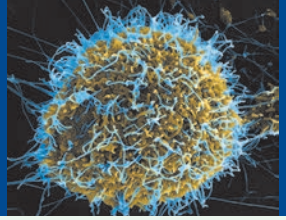

CASE STUDY 3: MYSTERIOUS RASHES

The headline in the February 6, 2002, edition of *the Seattle Times* read, "Mysterious Rash Hits School Kids Here, Across U.S." From October 2001 to June 2002, a total of 27 state health departments reported multiple groups of schoolchildren who developed mysterious rashes. The children complained of itchy, sunburn-like rashes on their cheeks and arms that lasted from a few hours to 2 weeks. Other symptoms included a burning sensation on the skin and hives that moved from one part of the body to another. A few children experienced other signs and symptoms, such as fever, vomiting, sore throat, or headache. The outbreaks affected 10–600 people at a time. A few teachers and school staff were affected, but rarely parents or siblings. Some schools were temporarily closed to clean air filters and check ventilation systems. Authorities at all of the schools that were closed reported an "exceptionally high" level of dust and dandruff in the air.

The level of parental concern and media hype prompted an investigation by the CDC. They found no common cause for the outbreaks, but there were a few reports of cases associated with parvovirus B19 infection (Fifth disease). The majority of cases remain unexplained. The CDC is continuing to monitor reports of groups of children with rashes and is providing technical assistance to state and local health departments investigating the outbreaks.

1. Rashes can be explained by a variety of causes. List a few possible causes.
2. Could additional cases be caused by a new or yet-to-be-identified virus? Why or why not?
3. The CDC experts pointed to several challenges that impeded their investigation of reported rashes among schoolchildren and the identification of their causes. List these challenges.

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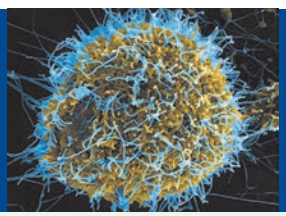

CASE STUDY 4: HUMAN METAPNEUMOVIRUS AT A DAY CARE FACILITY

The campus day care recently closed during the peak of the winter flu season because many of the young children were sick with lower respiratory tract infections. An announcement by e-mail was sent to all students, faculty, and staff at the college stating that the closure was due to a **metapneumovirus** outbreak. The announcement briefed the campus community with information about human metapneumonoviruses (hMPVs).

The announcement stated that hMPV was a newly identified respiratory tract pathogen discovered in the Netherlands in 2001. New tests confirmed that it is one of the most significant and common viral infections in humans. It is clinically indistinguishable from a viral relative known as respiratory syncytial virus (RSV). Both RSV and hMPV infections occur during the winter. hMPV may account for 2–12% or more of previously unexplained pediatric lower respiratory infections for which samples are sent to diagnostic laboratories, and a lesser percentage in adults.

Both hMPV and RSV cause upper and lower respiratory tract infections associated with serious illness in the young, immunosuppressed, elderly, and chronically ill. Common symptoms include cough, fever, wheezing or exacerbation of asthma, and **rhinorrhea** (runny nose). Severe symptoms may result in the need for intensive care admission and ventilator support. Healthy adults can get a mild form of the disease that is characterized by a cough, hoarseness, congestion, runny nose, and sore throat.

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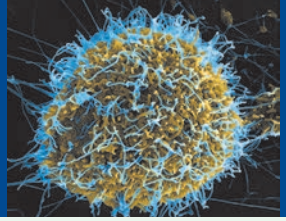

CASE STUDY 4: HUMAN METAPNEUMOVIRUS AT A DAY CARE FACILITY (continued)

Members of the campus day care staff were doing their best to limit the epidemic spread of the hMPV outbreak at the center. Primary care physicians need up-to-date knowledge and heightened awareness to recognize this new viral disease in patients.

1. hMPV and RSV belong in the *Paramyxoviridae* family. What type of nucleic acid genome do they have? Do they encode their own viral polymerase? (*Hint*: See http://viralzone.expasy.org.)
2. Draw a diagram of the replication cycle of hMPV.
3. How many genes does hMPV have in its genome?
4. The first report of a fatal encephalitis case associated with hMPV was published in the CDC's *Emerging Infectious Diseases* in 2005. The authors recommended screening for patients, especially children with encephalitis symptoms of unknown origin. What is encephalitis?
5. Have neurological symptoms been associated with other viruses in the *Paramyxoviridae* family? If so, which ones?

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Resources

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