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3

Virus Morphology, Replication, and Assembly

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I. Glossary of Abbreviations and Definitions
II. Introduction
III. Chemical Composition
  A. Viral Nucleic Acid (Genome)
  B. Viral Proteins
IV. Morphology
  A. Helical Capsids
  B. Icosahedral Capsids
V. Viral Replication Cycle
  A. Adsorption
  B. Penetration and Uncoating
  C. Targeting Viral Nucleocapsids to the Replication Site
  D. Post-Uncoating Events
  E. Transcription of Viral Genes
  F. Translation
  G. Replication of Viral Genome
VI. Assembly and Morphogenesis of Virus Particles
  A. Assembly of Naked Viruses
  B. Assembly of Enveloped Viruses
  C. Role of Viral Budding in Viral Pathogenesis
VII. Conclusions
  References

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I. GLOSSARY OF ABBREVIATIONS AND DEFINITIONS

Ambisense RNA: These RNAs are of partly positive-sense and partly negative-sense polarity.

Capsid coat or shell: The protein shell in contact or directly surrounding the viral nucleic acid (genome).

Capsomeres: These are morphological units that form capsids. Capsomeres consist of oligomers of one or more viral proteins.

CPE: Cytopathic effect; could be due to apoptosis, necrosis, or syncytium formation.

cRNA: Full-length plus-strand template RNA complementary to the minus-strand genomic RNA.

CTL: Cytotoxic T lymphocytes.

DI: Defective interfering viruses. DI virus particles contain a smaller viral genome, are noninfectious, and need the help of infectious (wild-type) virus for replication but, in turn, interfere with replication of homologous infectious (standard) viruses.

Ectodomain: The portion of the transmembrane protein that remains exposed outside of the cell or virus particle.

EIS: These are the *cis* elements of the unsegmented minus-strand RNA genome (e.g., VSV). "E" denotes the end or transcription termination and polyadenylation sequence; "I" stands for intragenic sequence not transcribed in messenger RNA (mRNA); "S" indicates the start sequence for the next mRNA.

Endocytic pathways (endocytosis): The process of internalization of external macromolecules or viruses, which involves specific binding to cell surface receptors. Viruses use this mechanism to enter into host cells. In this process, clathrin-coated vesicles and subcellular organelles like endosomes and lysosomes are involved.

Envelope: The viral membrane containing the lipid bilayer and associated proteins, which surround the nucleocapsid of enveloped viruses and form the outermost barrier of the enveloped virus particle.

Enveloped viruses: Viruses that possess an envelope or membrane surrounding the nucleocapsid. For enveloped viruses, the naked nucleocapsid is not infectious.

Episomal, extrachromosomal: The state of existence of nucleic acid molecules that do not became integrated into host cell chromosomes. They exist and multiply independently within the cell nucleus or cell cytoplasm.

Escape mutants: Virus mutants that are not neutralized by antibodies. These viruses possess amino acid change in the epitope and therefore no longer bind to the neutralizing antibody.

Exocytic pathways (exocytosis): The mechanism for transporting intracellular transmembrane or secretory proteins from intracellular compartments to the cell surface or extracellular environment. In this process, various subcellular compartments, like endoplasmic reticulum (ER) and Golgi complexes, are involved in protein transport.

Genome: The complete genetic information (DNA or RNA) of an organism.

Glycosylation: In this process, one or several carbohydrate groups are attached to proteins during their transport through the exocytic pathways. Sugar residues are attached at specific sites to amino acids such as serine or threonine (for o-linked) or asparagine (for N-linked) carbohydrate moieties. These carbohydrate moieties are also called glycans.

GSL: Glycosphingolipid.

HBV: Hepatitis B virus.

Helical capsids: These structures are spiral, springlike, and flexible rods. The RNA genome in a helical capsid is either exposed (influenza viruses) or enclosed (paramyxoviruses, rhabdovirus) by the nucleoprotein molecules constituting the nucleocapsid.

H1N1, etc.: Subtype specificity of influenza type A viruses. H denotes hemagglutinin (H1–H14), and N stands for neuraminidase (N1–N9) subtypes.

HIV: Human immunodeficiency virus.

HSV: Herpes simplex virus.

HRV-14: Human rhinovirus strain 14.

ICAM-1: Intracellular adhesion molecule-1, the receptor for rhinoviruses.

Icosahedron, icosadeltahedron, icosahedral symmetry, icosahedral capsids: Icosahedron is a structure with a twofold, threefold, and fivefold rotational symmetry. It is a polyhedron with 20 faces, 12 vertices, and 30 edges. Most icosahedral viruses have 60 (multiple of 60) subunits (e.g., polioviruses, togaviruses).

Inclusion bodies: Microscopic structures, produced in some virus-infected cells consisting of viral proteins, nucleic acids, and cellular elements (particularly cytoskeletal elements). Inclusion bodies can be intranuclear (herpesviruses), intracytoplasmic (paramyxoviruses).

IRES: Internal ribosome entry site.

LB: Lateral bodies found in poxviruses.

LCMV: Lymphocytic choriomeningitis virus, a member of the Arenavirus group.

LT: Large T antigen (ST = small T antigen) of SV40.

MOI: Multiplicity of infection, that is, infectious units adsorbed per cell.

Naked or non-enveloped viruses: These viruses do not have any membrane and the nucleocapsids represent the infectious virus.

NP: Nucleoprotein.

NTS, NLS: Nuclear targeting (or localizing) signal.

Nucleocapsid: The complete nucleic acid–protein complex of a virus particle. Sometimes the term viral ribonucleoprotein (vRNP) is used to indicate nucleocapsid (e.g., vRNP of influenza viruses).

ORF: Open reading frame.

Panhandle: A circular nucleic acid structure of single-stranded (ss) DNA or RNA with a double-stranded stem at the end produced by intrastrand hybridization due to partial complementarity of the nucleic acid sequences at both the 5′ and 3′ termini of ssRNA or DNA. The panhandle structures function as the promoter and are important for transcription and replication.

pfu: Plaque-forming unit.

Phagocytosis, viropexis: Uptake of particles by cells not totally dependent on receptor-mediated endocytosis. The particle on the surface is engulfed by the cell membrane into a phagocytic vesicle. These phagocytic vesicles then undergo similar changes as the endosome. Poxviruses enter cells by phagocytosis.

Poly(A): Polyadenylation at the 3′ end of an RNA molecule.

Protomer: The term often used to indicate a structural unit containing one or more nonidentical protein subunits. Promoters are used as a building-block for virus capsid assembly.

RDRP: RNA-dependent RNA polymerase, also called RNA transcriptase and RNA replicase.

RNA of positive and negative polarity: The RNA strand of the same polarity as the mRNA-encoding proteins is called positive-, plus-, or + strand RNA. When the RNA is of polarity opposite to the mRNA (i.e., cannot code for a protein), it is called negative-, minus-, or – strand RNA.

RNP: Ribonucleoprotein.

RSV: Respiratory syncytial virus.

RT: Reverse transcriptase, RNA-dependent DNA polymerase.

ST: Small T antigen (LT = large T antigen) of SV40.

Structural and nonstructural proteins: Structural proteins are those proteins that are found in virions either as components of capsid or envelope. Nonstructural proteins are those virally encoded proteins produced in the infected cells but not found in virions. Nonstructural proteins are usually catalytic and regulatory in nature and are also involved in modifying host functions.

Synchronous infection: When all cells in the culture are infected simultaneously. Cells are infected at a high MOI (>5) and at low temperatures (4°C). Then the temperature is raised to 37°C to permit entry and uncoating of all cell-bound viruses at the same time.

Syncytium (multinucleated giant cells): Cells possessing multiple nuclei are formed due to fusion among a number of cells. Usually viruses that can undergo fusion at a neutral pH (paramyxoviruses, retroviruses) produce syncytium.

Temperature-sensitive (ts) mutant: A mutant virus that will replicate at a permissive (low) temperature but not at the nonpermissive or restrictive (high) temperature. This phenotype is usually caused by missense mutations of one or more nucleotides, causing alteration of amino acid(s) of a protein that cannot assume the functional configuration at the nonpermissive (restrictive) temperature.

TGN: Trans-Golgi network.

Transmembrane proteins: These are membrane proteins that are anchored to the membrane by spanning the lipid bilayer of the membrane via transmembrane domains. These proteins can be classified as type I (e.g., influenza virus HA), type II (e.g., influenza virus NA), type III (e.g., influenza virus M2), or complex (e.g., coronaviral E1) depending on the orientation of the NH_2 and COOH termini (type I, II, or III), cleavage of signal peptide (type I), and multiple transmembrane spanning domains (complex).

Virion: The entire virus particle. It usually refers to infectious or complete virus particle as opposed to noninfectious or defective virus particles.

VSV: Vesicular stomatitis virus.

WSN/33 (H1N1): A neurotropic variant of WS/33 (H1N1), a human influenza virus isolated in 1933 (Francis and Moore, 1940).

II. INTRODUCTION

Viruses are unique life forms different from all other living organisms, either eukaryotes or prokaryotes, for three fundamental reasons: (1) the nature of environment in which they grow and multiply, (2) the nature of their genome, and

(3) the mode of their multiplication. First, they can function and multiply only inside another living organism, which may be either a prokaryotic or eukaryotic cell depending on the virus. Viruses are acellular and metabolically inert outside the host cell and are obligatory parasites. Although there are other examples of obligatory parasites among the eukaryotes and prokaryotes, the nature of the intimate relationship between viruses and their host (i.e., environment) is much different. For example, some viruses extend their parasitic behavior to another level of mutual coexistence with their host, that is, they not only exist intracellularly but can, and do in some cases, integrate their genome into the genome of their host and thus tie their fate to the fate of the host. In fact, under these conditions, the integrated viral genome behaves like a host gene(s), undergoing similar regulatory control in transcription and replication and similar evolutionary changes as do the host gene(s). Second, whereas all other living forms can use only DNA (and not RNA) as their genetic material (genome) for information transmission from parent to progeny, viruses can use either DNA or RNA as their genome, that is, some viruses can use only RNA (and not DNA) as their genetic material. Therefore, these classes of RNA viruses have developed new sets of enzymes for replicating and transcribing RNA from an RNA template, as such enzymes (RNA-dependent RNA polymerase or RDRP) are not normally found in either eukaryotic or prokaryotic cells. Finally, all eukaryotic and prokaryotic cells divide and multiply as a whole unit, that is, $1 \rightarrow 2 \rightarrow 4 \rightarrow 8$ and so on. However, viruses do not multiply as a unit. In fact, they have developed a much more efficient way to multiply just as complex machines are made in a modern factory. Different viral components are made separately from independent templates, and then these components are assembled into the whole and infectious units, also called virus particles (virions), just as the complex machines are efficiently assembled from individual components. In this chapter, I will discuss aspects of viral morphology, the mode of viral replication, and viral morphogenesis.

Viruses are a heterogenous group of microorganisms that vary with respect to size, morphology, and chemical composition. The size of virions ranges from 20 nm (parvovirus) to ~300 nm (poxvirus) in diameter, as compared to the size of *Escherichia coli*, which is about 1000 nm in length. Viral shape also varies. Some viruses are round (spherical), others filamentous, and still others pleomorphic. Usually, naked (non-enveloped) viruses have specific shapes and sizes, whereas some enveloped viruses (particularly enveloped viruses possessing helical nucleocapsids) are highly pleomorphic (e.g., orthomyxoviruses), with shapes varying from spherical to filamentous (Table I).

III. CHEMICAL COMPOSITION

The chemical composition of a virus depends on the nature of that virus, that is, the nature of the viral genome (RNA or DNA), the composition of the protein shell

TABLE I

Properties of the Virions of the Major Genera of DNA and RNA Animal Viruses

Viruses	Genome nature	Envelope	Shape	Genome polarity	Size (nm)	Transcriptase in virion	Symmetry of nucleocapsid
RNA viruses							
Enterovirus	S,[b] 1[a]	–	Icosahedral	+	~20–30	–	Icosahedral
Rhinovirus	S, 1	–	Icosahedral	+	20–30	–	Icosahedral
Calicivirus	S, 1	–	Icosahedral	+	20–30	–	Icosahedral
Alphavirus	S, 1	+	Spheroidal	+	50–60	–	Icosahedral
Flavivirus	S, 1	+	Spheroidal	+	40–50	–	Icosahedral
Orthomyxovirus	S, 8	+	Spheroidal[h]	–	80–120	+	Helical
Paramyxovirus	S, 1	+	Spheroidal	–	100–150	+	Helical
Coronavirus	S, 1	+	Spheroidal	+	80–220	–	Helical
Arenavirus	S, 2	+	Spheroidal	±[c]	85-120	+	Helical[d]
Bunyavirus	S, 3	+	Spheroidal	±[c]	90–100	+	Helical[d]
Retrovirus	S, 1[g]	+	Spheroidal	+	100–120	+[f]	Icosahedral[i]
Rhabdovirus	S, 1	+	Bullet-shaped	–	175 × 70	+	Helical
Reovirus	D, 10	–	Icosahedral	±	70–80	+	Icosahedral
Orbivirus	D, 10	–	Icosahedral	±	50–60	+	Icosahedral
Filovirus	S, 1	–	filamentous	–	≥80 × 800	+	Helical

continued

DNA viruses							
Papillomavirus	D, circular	–	Icosahedral	±	55	–	Icosahedral
Polyomavirus	D, circular	–	Icosahedral	±	45	–	Icosahedral
Adenovirus	D, linear	–	Icosahedral	±	70–80	–	Icosahedral
Herpesvirus	D, linear	+	Spheroidal	±	150	–	Icosahedral
Iridovirus[e]	D, linear	+[e]	Spheroidal	±	125 × 300	+	Icosahedral
Poxvirus	D, linear	+	Brick-shaped	±	300 × 240 × 140[j]	+	Complex
Parvovirus	S, linear	–	Icosahedral	+, –[k]	20	–	Icosahedral

[a]Genome, the number indicates the segments of RNA present in the virus particle. All RNA genome is haploid except retrovirus (diploid).

[b]D = double-stranded; S = single-stranded.

[c]Ambisense (contains coding for protein on both genomic and complementary RNA strands).

[d]Circular helical nucleocapsid.

[e]Insect iridoviruses have no envelope; vertebrate members are enveloped.

[f]Reverse transcriptase (RT).

[g]Diploid, two molecules of the same RNA (+ strand) segment are present in one virus particle.

[h]Pleomorphic including filamentous forms.

[i]The capsid structure of mature retroviruses is not fully known, although it appears icosahedral.

[j]Length × width × thickness.

[k]Some virus particles contain (+)-strand and others contain (–)-strand DNAs.

called the viral "nucleocapsid" surrounding the genome, and the presence or absence of viral membrane depending on whether the virus is enveloped or naked. All viruses have nucleocapsids and therefore contain nucleic acids and proteins. The nucleic acid is the genome that contains the information necessary for viral function and multiplication, and this information is passed from the parent to progeny viruses. Some viruses contain extragenomic nucleic acid, for example, tRNA in retroviruses and ribosomal RNA in arenaviruses. Viral proteins have three primary functions. They (1) provide the shell to protect the nucleic acid from degradation by environmental nucleases, (2) facilitate transfer of the genome from one host to another, and (3) provide many of the enzymatic and regulatory functions needed for transcription and replication so that viruses can survive, multiply, and perpetuate. In addition to the capsid shell, many viruses also possess an envelope (or viral membrane) around the nucleocapsid. The envelope in these viruses is critical for transmission of those viruses from one host to another host. The naked nucleocapsids of enveloped viruses are noninfectious because they lack the receptor binding protein. The viral envelope contains lipids and carbohydrates in addition to "envelope- or membrane-associated" viral proteins. The viral genome codes for most, if not all, of the proteins associated with the viral envelope. Lipids of the viral membrane are synthesized by the host cell and derived from the host cell. Therefore, viral lipid composition varies depending on the host cell in which the virus grows and also on the type of the cellular membrane (e.g., ER, Golgi, plasma or nuclear membrane) from which the particular type of virus buds. The carbohydrate content of the viral envelope is usually determined by the nature of glycosylation (N-glycosylation, O-glycosylation, complex versus simple sugar addition) of the viral envelope proteins, which may in turn undergo other modifications such as myristylation, palmitoylation, sulfation, and phosphorylation.

A. Viral Nucleic Acid (Genome)

Genomes of different viruses are widely diverse in size and complexity. Some are comprised of DNA, others of RNA. As mentioned earlier, only in viruses is RNA known to function as a genome. Viral DNA genomes vary in complexity ranging from 5 kb containing 5–6 genes (parvoviruses, SV40) to 300 kb (avipoxviruses) containing more than 200 genes. Some DNA genomes are double-stranded (SV40), some are partially double-stranded (hepatitis B virus), and still others are single-stranded (parvoviruses) (Table I and II). The single-stranded viral DNAs can be of either plus or minus polarity. Some DNA genomes are circular (and supercoiled), while others are linear. Some linear DNA genomes become circular intermediates during replication. Many viral DNA genomes are terminally redundant in their nucleotide sequences.

RNA genomes of viruses also vary in complexity but not as widely as do DNA genomes. They range from ~7 kb (rhinoviruses) to ~30 kb (coronaviruses). Coro-

TABLE II

Replication of DNA Viruses

Virus	Form of DNA	Polymerase	Activity	Presence in virion	Replication site in cell
Papovaviruses	ds[a]	Host	DNA *pol*	–	Nucleus
Adenoviruses	ds	Viral	DNA *pol*	–	Nucleus
Herpesviruses	ds	Viral	DNA *pol*	–	Nucleus
Poxviruses	ds	Viral	DNA *pol*	–[b]	Cytoplasm
Parvoviruses	ss	Host	DNA *pol*	–	Nucleus
Hepadnaviruses	Partially ds	Viral	Reverse transcriptase (RT)	+	Nucleus/ cytoplasm

[a]ds = double-stranded; ss = single-stranded.

[b]Virions contain DNA-dependent RNA transcriptase and many other enzymes, but not DNA-dependent DNA polymerase.

naviral RNA represents the largest stable single-stranded RNA found in nature. Viral RNA can be single- or double-stranded (Tables I and III).

The viral RNA genome may be unsegmented, consisting of a single RNA molecule, or segmented, consisting of multiple segments. Usually, viral genomes are haploid, but some are diploid (e.g., retroviruses; Fig. 1). Some viral RNA genomes may be linear, whereas others have partial terminal complementarity assuming panhandle structures (e.g., orthomyxoviruses). Some of the single-stranded RNA genomes are of plus or "positive" polarity, meaning that they can be translated directly into proteins, and others are of minus or "negative" polarity, meaning they must be used as a template to synthesize a translatable complementary strand (mRNA), and still others are ambisense (Table I). The plus-polarity naked viral genomes (except for retroviruses), completely free from all viral proteins, are infectious when introduced into a permissive cell, whereas minus-polarity naked genomes are noninfectious. Viruses possessing the minus-polarity genome therefore must carry an enzyme, RNA-dependent RNA polymerase (RDRP), inside the virus particle in order to initiate the infectious cycle. Similarly, retroviruses must possess reverse transcriptase (RT, RNA-dependent DNA polymerase) to initiate the infectious cycle inside host cells. However, using reverse genetics, many of the RNA genomes of both plus and minus polarity can be converted into infectious double-stranded DNA, thus permitting artificially induced mutational changes and genetic analysis of the viral genome, as well as use in DNA vaccination and gene therapy. Some of the DNA (adenoviruses, hepadnaviruses) as well as RNA (polioviruses) viral genomes possess a covalently linked terminal protein at the 5′ end of a genomic nucleic acid strand, which

TABLE III

Replication of RNA Viruses

	Virus	Form of RNA	Source of nucleic polymerase	Nature of polymerase activity	Presence of polymerase in virion	Viral replication site within host cell
A	Paramyxovirus, Rhabdovirus	ss[a] (−), unsegmented	Viral	RNA-dependent RNA polymerase (RDRP)	+	Cytoplasm
B.	Bunyavirus, Arenavirus	ss[c](±), segmented	Viral	RDRP	+	Cytoplasm
C.	Orthomyxovirus (Influenza virus)	ss (−), segmented	Viral	RDRP	+	Nucleus
D.	Rotavirus, Reovirus, Orbivirus	ds[b](±), segmented	Viral	RDRP	+	Cytoplasm
E.	Picornavirus (Poliovirus, Hepatitis A), Togavirus (Sindbis virus), Coronavirus	ss (+), unsegmented	Viral	RDRP	−	Cytoplasm
F.	Retrovirus, (HIV)	ss (+), unsegmented, diploid	Viral	Reverse transcriptase	+	Nucleus

[a]ss = single-stranded.

[b]ds = double-stranded; + or − indicates positive or negative polarity.

[c]± = ambisense genome.

provides critical functions for initiating DNA or RNA replication. Some positive-strand RNA viral genomes are also capped at the 5′ end and polyadenylated at the 3′ end (togaviruses), while others are not capped at the 5′ end (polioviruses) but possess polyadenylation (poly(A)) at the 3′ end. The minus-strand RNA genomes do not possess either the cap at the 5′ end or poly(A) at the 3′ end. Usually, the 5′ and 3′ ends of the minus-strand RNA genome are partially complementary, often forming panhandles by intrastrand hybridization and function as their own promoters for transcription and replication.

Organization of genes in the RNA genome varies between different groups of viruses. For positive-strand naked RNA viruses (e.g., polioviruses), which are

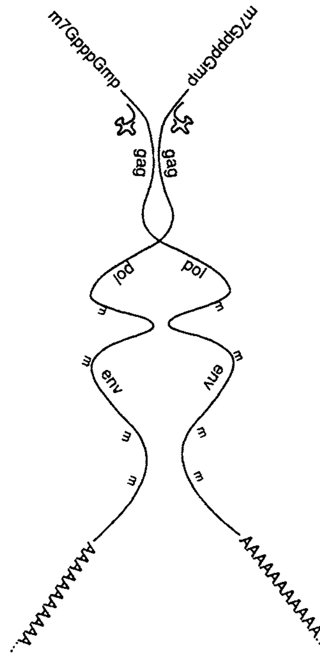


Fig. 1. Features of the retrovirus genome. The diploid RNA genome includes the following from 5′ to 3′: the m^7Gppp capping group, the primer tRNA, the coding regions, M_6A residues (m), and the 3′ poly(AAAAA) sequence. Reprinted with permission from Fields and Knipe (1990).

translated into a single large polyprotein, the 5′ end of the genome is not capped but is rather covalently linked to a small protein, VPg (Fig. 2). The 5′ end of these viral genomes contains an untranslated region possessing a highly ordered secondary structure for internal ribosome entry, followed next in sequence by the genes of capsid proteins (VP4, VP2, VP3, VP1). The genes for nonstructural proteins including proteases and viral replicase (an RNA-dependent RNA polymerase) are located in the 3′ half of the genome. However, for the plus-strand enveloped RNA viruses (e.g., Sindbis viruses), the genes for the nonstructural proteins are present at the 5′ end and structural proteins including capsid and envelope proteins are present in the 3′ half of the genome. Structural genes of this latter type of viruses are translated from a separate subgenomic mRNA, whereas their nonstructural proteins are translated from the genomic RNA. The large plus-strand coronavirus RNA genome possesses the nonstructural genes in the 5′ half and structural genes in the 3′ half of the genome. The gene for the highly abundant nucleoprotein (N protein) of coronaviruses is present at the 3′ end of the genome.

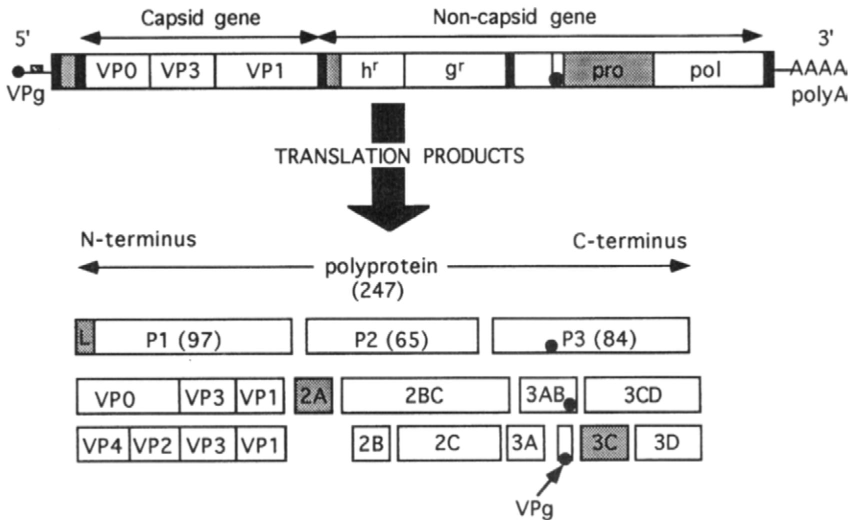


Fig. 2. Organization of picornaviral genome (RNA) and its translation products. P1, P2, and P3 indicate three intermediate precursor proteins cleaved from the polyprotein. These precursor proteins are further cleaved by virus-encoded proteases into mature functional proteins. Numbers in parentheses indicate molecular weights in thousands. h^r and g^r indicate host range and guanidine resistance determinants, respectively. 2A and 3C are proteinases involved in cleavage of the polyprotein and precursor proteins into mature viral proteins. Vpg, VP0, etc. indicate specific viral proteins.

For unsegmented minus-strand RNA genomes, the order of genes for both rhabdo- and paramyxoviruses are similar. Structural genes for capsid (N and P proteins) and envelope proteins are at the 3′ half, and the large polymerase (L) gene occupies the entire 5′ half of the minus-strand RNA genome (Fig. 3). The 3′ end of the template (minus-strand) RNA is transcribed into a leader (ℓ) sequence that is not present in the mRNA, and the region between two genes is separated by an element called the EIS. It consists of an "E" (end) sequence for transcription termination and polyadenylation of a gene, an "I" (intergenic) sequence that allows the viral transcriptase to escape (therefore the "I" sequence is not represented in the mRNA), and "S" (the start) sequences, which denote the start of the next gene. EIS sequences in the genome vary for different viruses in these groups.

B. Viral Proteins

Proteins are major constituents of the viral structure, and their main functions, as indicated earlier, are to protect the nucleic acid from nucleases and to provide

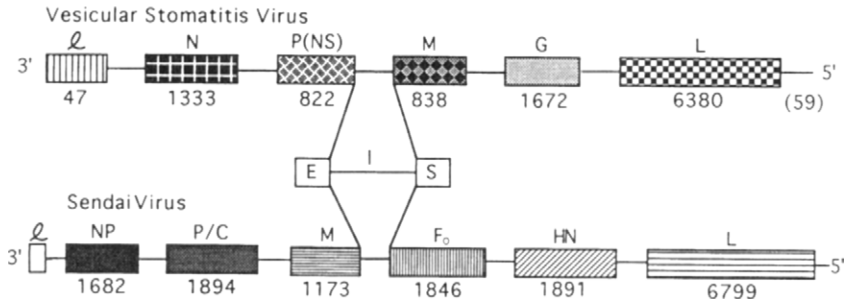


Fig. 3. Genome of unsegmented negative-strand RNA viruses (vesicular stomatitis virus [VSV] and Sendai viruses). Numbers underneath rectangles represent the number of nucleotides in each gene (shown above the line). ℓ = leader sequence; E = end (or transcription termination) sequence; I = intergenic sequence (not transcribed); S = start sequence of mRNA of the next gene; N, NP = nucleoproteins; P/C, P (NS) = phosphoprotein; M = matrix protein; G, F, HN = glycoproteins; L = polymerase protein.

receptor-binding site(s) for virus attachment, which is required for efficient transmission of virus from one host to another. Viral proteins can be classified as either nonstructural or structural. Nonstructural proteins are those encoded by the virion genome, and expressed inside the virus-infected host cells, but not found in the virion particles. These nonstructural proteins usually have regulatory or catalytic functions, which are involved either in viral replication or transcription processes or are involved in modifying host functions. Structural proteins are broadly defined as those proteins found in virus particles. The majority of these structural proteins constitute the viral capsid or core, and are intimately associated with the viral genome to form the nucleocapsid. The cores of some viruses also contain regulatory or catalytic proteins as minor structural proteins (e.g., proteins with enzymatic functions, such as transcriptase (RDRP) or reverse transcriptase (RT) (Tables I–III). In addition, some viruses include host proteins such as histones associated with the viral genome in virus particles (e.g., minichromosome in SV40) or ribosomes, as is the case in arenaviruses. Although these minor virus-coded and host-coded proteins are critically involved in virus replication and infectivity, they are not essential for formation of viral capsids.

In addition to having viral capsids, the enveloped viruses possess membranes (or envelopes) surrounding the viral capsids. These viral membranes, as noted earlier, contain lipids derived from the host membrane and proteins specified by the viral genome. Two types of proteins are found in the viral membrane: transmembrane proteins and matrix proteins.

1. Transmembrane Proteins

Transmembrane proteins, which can be either type I (such as influenza virus hemagglutinin), type II (such as influenza virus neuraminidase), and type III (such as influenza virus M2) depending on their molecular orientation; or complex proteins, containing multiple transmembrane domains (such as E1 glycoprotein of coronaviruses). Enveloped viruses may contain either only one (as in the G protein of VSV), two (as in the HN and F proteins in paramyxoviruses) or multiple transmembrane proteins (as in the influenza viruses, herpesviruses, poxviruses, etc.) on their envelope. These transmembrane proteins are often glycosylated via N- or O-glycosidic bonds, and their carbohydrate moieties can be comprised of simple sugars, usually consisting of mannose molecules, or complex sugars, including galactose, glucosamine, galactosamine, fucose, and mannose, as well as also sialic acid residues. Proper glycosylation of viral proteins is often important to provide for the necessary molecular stability, solubility, oligomer formation, and intracellular transport of viral proteins, as well as for modulating the host immune response, including epitope masking and unmasking. These glycans also may be important in apical sorting of proteins within polarized epithelial cells. It often is the case that one or more of these transmembrane proteins are involved in providing important functions in the processes of receptor binding, fusing of the viral envelope, uncoating of the viral genome, releasing of mature viruses from the infected cells, and spreading of viruses from cell to cell (e.g., function of NA, the neuraminidase protein, in releasing influenza viruses). These envelope proteins are also important for host defense, where they elicit neutralizing antibodies as well as CTL response against the virus infection in infected hosts and therefore play a critical role in vaccination and protection against viral infections.

2. Matrix Proteins

In addition to the transmembrane proteins, the majority of these enveloped viruses also contain another type of membrane protein called a *matrix protein* (e.g., M1 protein of influenza viruses), which forms a shell underneath the membrane, enclosing the capsid. The matrix proteins are therefore likely to interact with the lipid bilayer and transmembrane proteins of the viral envelope on the outer side and with the nucleocapsid on the inner side. Matrix proteins are also usually the most abundant proteins in enveloped virus particles and are critical for the budding of enveloped viruses. Some enveloped viruses containing icosahedral capsids do not possess typical matrix proteins around the nucleocapsids (e.g., togaviruses).

IV. MORPHOLOGY

Viruses vary greatly in size and shape. They can be either spherical or cylindrical (rod-shaped) (Fig. 4) or even pleomorphic (Figs. 5 and 6). Primarily, the virus

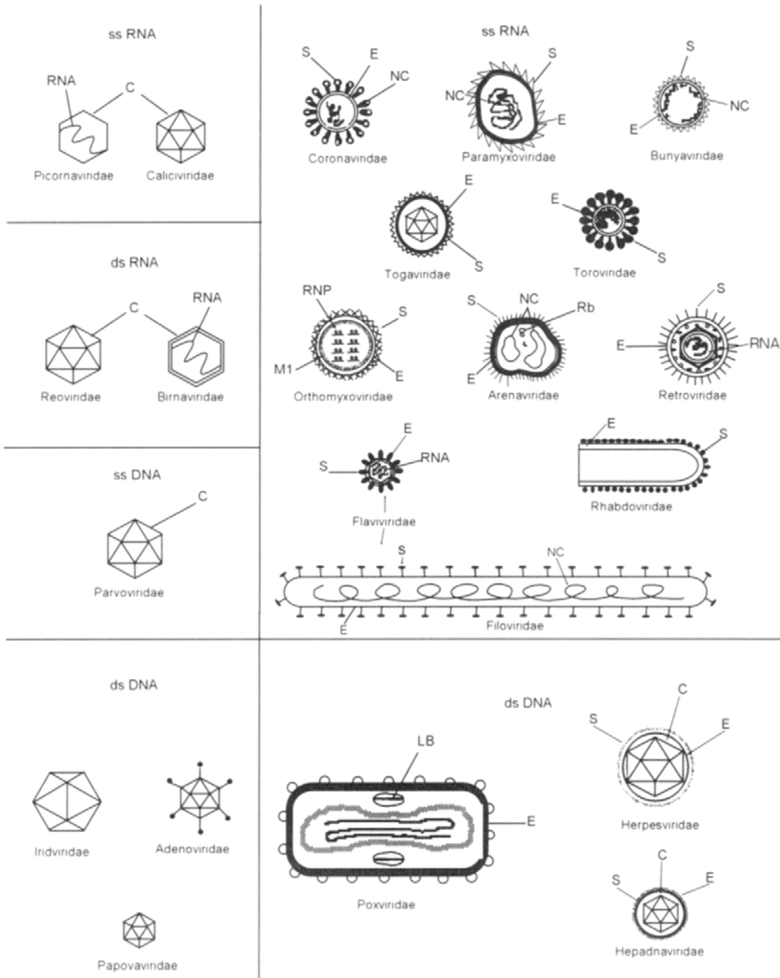
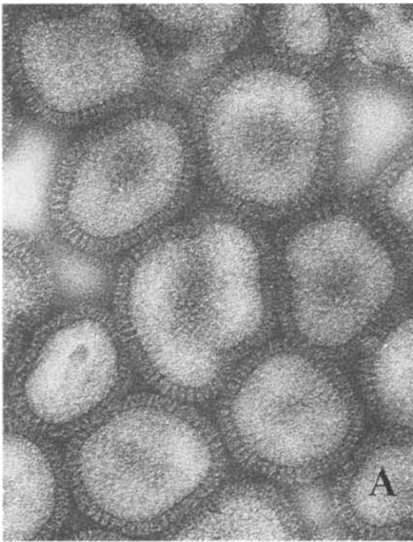
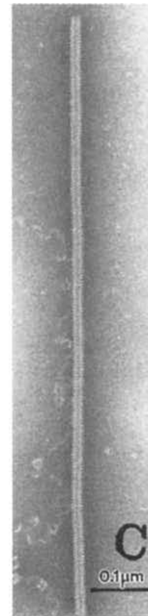


Fig. 4. Schematic presentation of different forms of viral structures. C = capsid; S = spike on viral envelope; E = viral lipid envelope; NC = nucleocapsid (i.e., capsid proteins in association with either RNA or DNA); M1 = matrix protein of influenza virus; LB = lateral bodies present in poxviruses; ss = single-stranded, ds = double-stranded RNA or DNA.





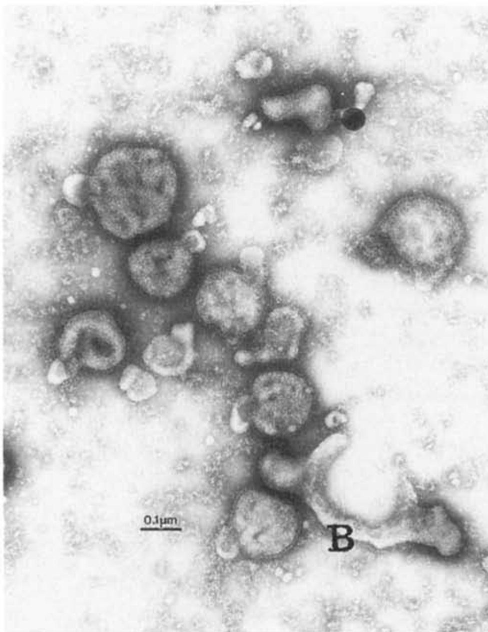


Fig. 5. Transmission electron micrographs of influenza virus (**A**), Sendai virus (**B**), and helical Sendai viral nucleocapsid (**C**). These electron micrographs were provided by and are reprinted with permission from K. G. Murti of St. Jude Children's Research Hospital of Memphis, Tennessee.

structure is determined by the nature of the capsid and whether the capsid is naked or surrounded by an envelope. The structure of the capsid is in part determined by the protein and nucleic acid (nucleocapsid) interactions, but principally by the protein–protein interactions of the capsid protein(s). In most cases, the nucleic acid is incorporated after the majority of the protein shell of the capsid has been formed, or capsids can remain empty, resulting in noninfectious virus particles. The capsids are composed of repeating protein subunits called capsomeres. Capsomeres are composed of multimeric units of either a single protein, or often heteromeric units of more than one protein.

The formation of the viral capsid and its shape is primarily determined by the three-dimensional structure of the capsid proteins, which in turn is determined by the specific amino-acid sequence encoded by the viral nucleic acid. The amino-acid sequence is considered the primary structure of the protein, whose three-dimensional structure is composed of secondary structures such as α helices, β sheets, and random coils. These secondary structures interact with each other, forming the tertiary and quaternary structures, which are usually stabilized by noncovalent interactions (sometimes by covalent disulfide linkages), and represent folding of the proteins into relatively stable structures of microdomains (e.g., globular heads). In addition, extended and flexible regions of the proteins, called hinges, are also present, and these hinges become important for interaction with other members of the protein subunits that form the capsomeres. In most viruses, contacts between capsomeres are repeated, exhibiting a symmetry. This is a process of self-assembly driven by the stability of interaction among the protein subunits forming the capsomeres and the capsomeres forming the capsid. Viral capsids have either a helical (springlike) or icosahedral-based (cuboidal or spherical) symmetry.

A. Helical Capsids

Helical capsids are usually flexible and rodlike. The length of the helical capsid is usually determined by the length of the nucleic acids, that is, some defective interfering (DI) viruses having shorter nucleic acids will have a shorter helical nucleocapsid (e.g., DI RNA of VSV). Helical capsids can be naked, that is, without an envelope (e.g., tobacco mosaic virus). However, there is no known example of an animal virus with a naked helical nucleocapsid. All animal viruses with helical capsids found to date are enveloped. However, such helical capsids when enclosed in an envelope can appear to be either rod-shaped (e.g., rhabdoviruses) or spherical (e.g., orthomyxo- or paramyxoviruses), indicating that the helical capsid in these viruses is flexible (Fig. 5). Some helical capsids can be further folded, forming

supercoiled nucleocapsids (e.g., orthomyxoviruses). Usually, enveloped viruses with helical capsids are pleomorphic, forming either spherical or filamentous particles (Figs. 5 and 6). Helical capsids can package only single-stranded RNA, but not double-stranded DNA or RNA, possibly because of the rigidity of the double-stranded nucleic acids. However, some viruses with helical capsids may possess only one capsid containing one virion RNA (unsegmented) molecule (rhabdoviruses, paramyxoviruses) or multiple capsids containing multiple RNA segments (orthomyxoviruses). Viruses containing multiple RNA segments can undergo reassortment with other related viruses, thus exchanging different RNA segments and giving rise to new viruses with different antigenic and virulence determinants (e.g., the antigenic shift that occurs in influenza viruses). The genomic RNA is protected by the helical capsid in some viruses (e.g., paramyxo- and rhabdoviruses) but remains exposed in others (e.g., orthomyxoviruses). A single viral protein (e.g., NP protein of orthomyxoviruses) is usually involved in helical capsid formation.

B. Icosahedral Capsids

Viruses with icosahedral capsids possess a closed shell enclosing the nucleic acid inside (Fig. 4). An icosahedron has 20 triangular faces, 30 edges, and 12 vertices and is characterized by a 5:3:2-fold rotational symmetry. Unlike helical nucleocapsids that package only single-stranded nucleic acid, icosahedral capsids can be used to package either single- or double-stranded RNA and DNA molecules. However, whereas either plus- or minus-strand DNAs are found in the icosahedral capsids of parvoviruses, there are as yet no examples of an icosahedral virus with minus-strand RNA. An icosahedral virus can be either naked or enveloped; but, unlike the helical enveloped viruses, the enveloped icosahedral viruses are less pleomorphic in their shape because the icosahedron capsid structure is rather rigid, and in addition, with icosahedral capsids, the overall size is fixed for a particular virus. The virus particle's formation, stability, and size do not depend on the amount of nucleic acid in the capsid. Although the packaging of the nucleic inside the icosahedral capsid is relatively fixed and does not vary greatly, noninfectious viruses containing empty capsids (i.e., without nucleic acid), can often be seen in virus populations. In recent years, the complete three-dimensional structures of several icosahedral viruses have been determined at the atomic level using the powerful tools of cryoelectron microscopy and X-ray diffraction analysis. Such analyses have led to the rational design of a number of antiviral drugs. Some examples of such three-dimensional viral structures are presented in Figure 7.

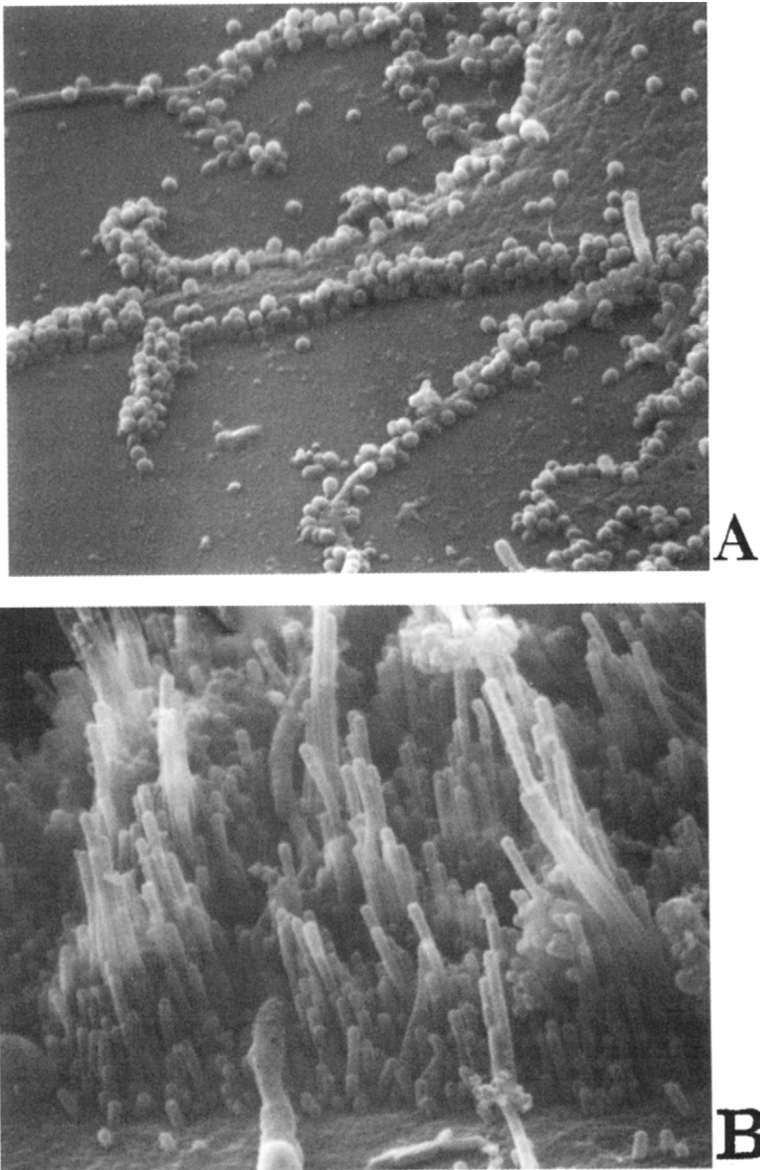


Fig. 6. Scanning electron micrographs of influenza viruses budding from infected cells. Spherical (**A**) and filamentous (**B**) forms are seen (×40,000). These micrographs were provided by and are reprinted with permission from David Hockley of the National Institute for Biological Standards and Control at Hertfordshire, UK.

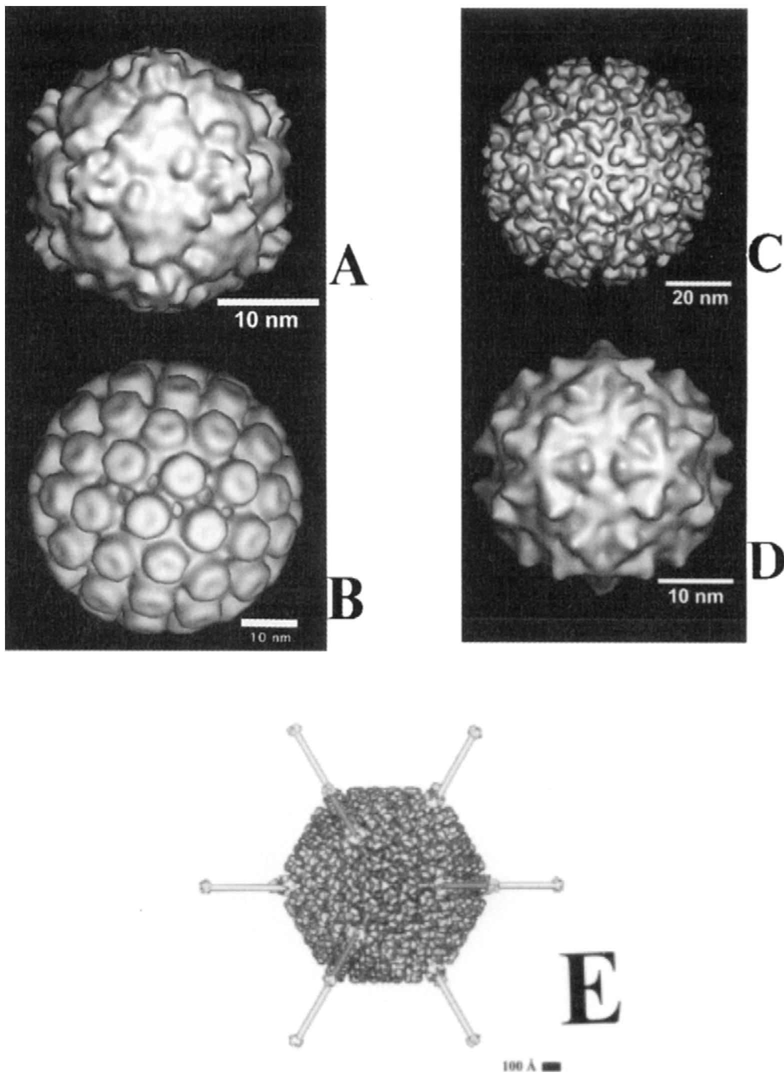


Fig. 7. Structure of representative RNA and DNA viruses as determined by cryoelectron microscopy: (**A**) human rhinovirus 14, an ssRNA virus; (**B**) SV40, a dsDNA virus; (**C**) Sindbis viral capsid, an ssRNA virus; (**D**) Flockhouse virus, a positive-strand bipartite ssRNA insect virus; (**E**) adenovirus, a dsDNA virus. The micrographs of human rhinovirus 14, SV40, Sindbis virus, and Flockhouse virus were provided by and are reprinted with permission from Norm Olson and Jim Baker of Purdue University. The adenovirus micrograph was provided by and is reprinted with permission from Phoebe Stewart of UCLA.

V. VIRAL REPLICATION CYCLE

To survive, viruses must multiply. Since viruses cannot multiply outside the host cell, they must infect host cells and use cellular machinery and energy supplies to replicate and produce the progeny viruses, which must in turn infect other hosts, and the cycle continues. Host–virus interaction at the cellular level is therefore obligatory for virus replication. Specific host cells can be either nonsusceptible (i.e., resistant or nonpermissive) or susceptible (i.e., permissive) to a particular virus. Nonsusceptibility of cells can be either at the attachment and entry phase (e.g., lack of a suitable receptor for a virus at the cell surface), at the intracellular phase (i.e., a block in synthesis of viral macromolecules), or at the assembly and exit phase. Following infection, viruses can cause abortive (nonproductive) or productive infection. Only productive infection yields infectious progeny virus particles. Following either abortive or productive infection, the host cell may survive or die (i.e., the cytopathic effect [CPE]). CPE caused by a virus does not necessarily indicate the permissiveness of a cell to a virus leading to productive infection. The viral genome in abortive infection may be degraded or may integrate into the host DNA or exist as extrachromosomal (episomal) DNA in the surviving cell. The growth properties of such cells may be altered, including the possibility that they may become transformed and cancerous. Alternatively, cells containing the integrated viral DNA may behave normally, exhibiting little change in their normal properties. Malignant transformation of the infected cells often depends on the site of viral genomic integration leading to activation of cellular oncogenes, disruption or inhibition of tumor suppressor genes, or synthesis of the viral oncogene products that are encoded by the virus in its genome. In the infected cells, the virus genome may remain dormant, resulting in a latent infection, and it can be activated later, producing infectious viruses, as occurs with herpesviruses. Alternatively, infected cells may yield virus at a low level without affecting cell survival, resulting in persistent infection, as occurs with LCMV.

The effect of virus infection has been studied at both the cellular and organismic levels. At the organismic level, it is called "viral pathogenesis," while at the cellular level it is called the "cytopathic effect" (CPE). Under these conditions, cells may undergo morphological changes, including rounding, detachment, cell death and cell lysis (either apoptotic or necrotic), and syncytium (giant multinucleated cell) formation as well as inclusion body formation. Many of these changes are caused by the toxic effects of viral proteins affecting host macromolecular synthesis, including DNA replication, DNA fragmentation, mRNA transcription, translation, protein modification, and degradation, as well as other cellular synthetic and catalytic processes. Furthermore, since the same cellular machineries are directed toward viral macromolecular synthesis, the host is deprived of their functions. In addition to direct cell killing, virus infection can indirectly cause

injury to tissues in a complex organism, as a result of complex host–viral immune interactions (i.e., immunopathology), as well as by cytokine production causing inflammatory reactions.

It is evident from the foregoing that, for successful replication of a virus, it must find susceptible host cells, and it must be able to attach itself to and penetrate into the host cell, and be uncoated, rendering the viral genome available for interaction of the viral and cellular machineries for transcription, translation, and replication of the viral genome. Finally, the newly synthesized viral components must be assembled into progeny viruses and released into the medium (outside environment) to infect other hosts. Whether with the cultured cells in laboratory or the complex organisms in nature, the virus–host interaction always occurs at the level of single cells. Thus, the viral infectious cycle (also known as the viral growth cycle, or replication cycle) can be divided into different phases, namely: (1) adsorption (attachment), penetration, and uncoating; (2) transcription, translation, and replication; and (3) assembly and release.

A. Adsorption

Viral adsorption is defined as the specific binding of a virus to a cellular (host) receptor. It is a receptor to ligand interaction in which viruses function as specific ligands and bind to the receptors present on the cell surface. Ligand functions of the virus are provided by the specific viral proteins present on the surface of the virus. For naked (i.e., non-enveloped) viruses, this function is performed by one of the capsid proteins, and for enveloped viruses, one of the membrane proteins functions as the ligand (variously also known as the receptor-binding protein, viral attachment protein or antireceptor) for the host receptor. Usually only one viral protein provides the receptor binding function, although one or more cellular proteins can function as receptor and coreceptor. For enveloped viruses, a classic example of a viral ligand (i.e., receptor-binding protein) is influenza virus hemagglutinin (HA), and its receptor-binding site is present on the globular head of the HA spike. For non-enveloped viruses, a classic example of a viral ligand is the VP1 of rhinoviruses. When five VP1 proteins are packed together within the viral capsid structure, the confluence of these grooves forms a depression called a canyon. The canyon has been shown to be the site for interaction between human rhinovirus-14 (HRV-14) and the cellular molecule ICAM-1 (receptor for rhinovirus). The amino acids lining the floor of these canyons are highly conserved, but residues on the surface of the canyon are variable (Fig. 8). Antibodies can bind to the surface epitopes around and in the proximity of the receptor-binding site and thus interfere with virus attachment by steric hindrance. Viruses can accept mutations in these surface epitopes and thereby escape (and are thus known as

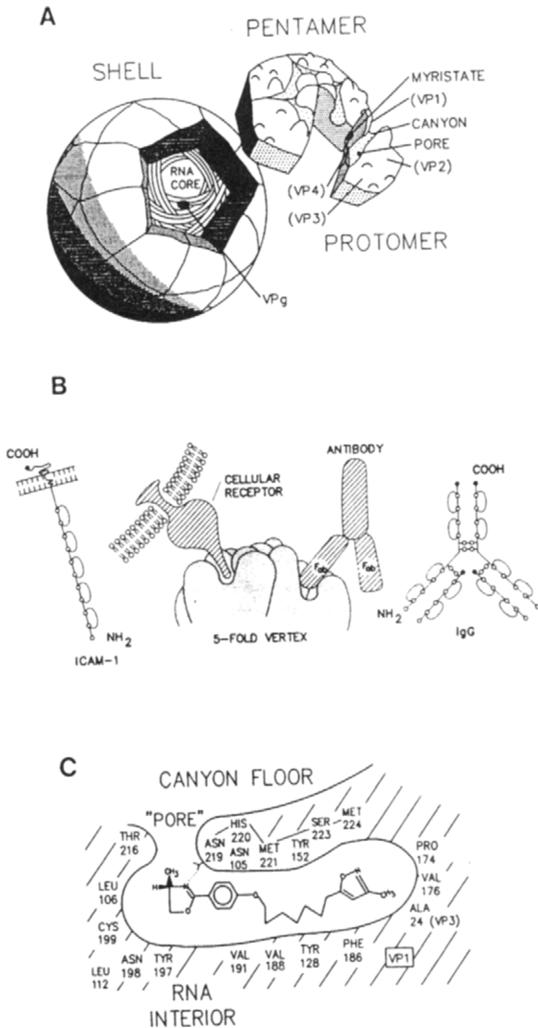


Fig. 8. Key features in the function of cellular receptor interactions with an invading virus, like a typical picornavirus. (**A**) Exploded diagram showing internal location at the canyon-like center of the pentamer fivefold vertex with myristate residues on the NH_2 terminus of VP4. (**B**) Binding of cellular receptor (ICAM-1 molecule) to the floor of the canyon. Note that the binding site of the ICAM-1 molecule, identified as a major rhinovirus receptor, has a diameter roughly half that of an IgG antibody molecule. (**C**) Location of a drug-binding site in VP1 of HRV14 (human rhinovirus 14) and identity of amino-acid residues lining the wall. The drug depicted here, WIN 52084, prevents attachment of HRV14 by deforming part of the canyon floor. The pentamer vertex lies to the right. Reprinted with permission from Fields and Knipe (1990).

escape mutants) neutralization by specific antibodies, but the receptor-binding site usually does not undergo mutational changes and remains conserved. This also appears to be the case with influenza virus hemagglutinin and other viral receptor-binding sites that remain conserved despite the variation in the neutralizing epitopes of the same viral protein. Thus, the viral receptor-binding site is usually a depression or canyon and is therefore protected from the mutational pressure of antibodies.

The cellular receptors of many viruses have been recently identified. Cellular receptors must be present on the cell surface and are either carbohydrates, lipids, or proteins. Sialooligosaccharides present on either glycoproteins or glycolipids function as receptors for orthomyxoviruses, paramyxoviruses, or polyomaviruses; phosphatidylserine and phosphatidylinositol are the likely receptors for VSV; and immunoglobulin superfamily molecules (CD4 for HIV, ICAM-1 for rhinovirus) as well as hormone or neurotransmitter receptors function as receptors for a number of other viruses (e.g., epidermal growth factor for vaccinia viruses, β-adrenergic receptor for reovirus, acetylcholine receptor for rabies virus). Some viruses have more than one receptor, one being the primary receptor and the other a coreceptor. A classic example of this is the case of CD4 and chemokine receptors (CXCR4, CCR5, etc.) respectively functioning as the receptor and coreceptor for HIV. Both the receptor and coreceptor are needed for productive HIV infection, although only one viral protein (gp120) provides the receptor-binding sites for both receptor and coreceptors. Receptor–virus interaction is a major reason for the host and tissue tropism of viruses. Recent studies have shown that lack of a specific coreceptor on a cell's surface provides resistance to HIV infection in some persons. Receptor–virus interactions are specific, and the noncovalent binding is independent of energy or temperature. Thus, the kinetics of viral binding to cells can be determined at 4°C, which serves as a research aid since their interaction at that temperature prevents viral penetration and uncoating. Therefore, binding virus to cells at 4°C and subsequently raising the temperature to 37°C can be used to infect cells synchronously and to study the subsequent events such as uncoating and penetration of virus into host cells. The time course of viral adsorption follows first-order kinetics and is dependent on virus-to-cell concentration. Usually, cells contain a large number of receptors: in the range of 10^4–10^5 per cell.

B. Penetration and Uncoating

Following specific ligand-to-receptor interaction, the next steps in virus replication include penetration of virus into the host cell and uncoating of the viral genome, which are energy-dependent processes and can be prevented experimentally in the laboratory by subjecting the virus–cell complex to low temperatures (4°C). Penetration refers to entry of the surface-bound virus particles inside the cell, where they either exist free in the cytoplasm or inside the host cell vesicles

(usually within endosomes). Quantitatively, penetration of virus particles is measured by the loss of the ability of antiviral antibodies to neutralize the cell-bound virus particles after adsorption, an effect that occurs because, after the viral particles have entered the cell, they are protected and no longer accessible to antibodies outside the cell. Uncoating, on the other hand, refers to disruption of virus particles, causing partial or complete separation of nucleic acid from the capsid, and is needed for initiation of transcription and translation of the viral genome. Uncoating can be assessed by, among other things, alterations in viral morphology or viral density, release of nucleocapsid and membrane proteins from enveloped virus particles, as well as by the accessibility of the viral genome to nucleases. For such viruses as orthomyxovirus and poliovirus these processes are separated temporally (i.e., penetration is followed by uncoating in the cytoplasm), but for others both penetration and uncoating occur simultaneously at the cell surface (e.g., paramyxoviruses, HIV). Uncoating refers to the step in which the viral genome becomes functional either transcriptionally or translationally. However, complete separation of nucleic acid from all capsid proteins is not required for most viruses. For naked viruses, uncoating is a postpenetration process that occurs either in the endosome or in the nucleus. Viruses that undergo uncoating in the cytoplasm following endocytosis require low pH (~5) in the endosome for uncoating, whereas viruses that undergo fusion at the cell surface can undergo uncoating in a pH-independent manner.

Naked viruses like the RNA-based picornaviruses enter into the cytoplasm of the infected cells via receptor-mediated endocytosis (Fig. 9) or by phagocytosis (viropexis). In the endosome, the virus particle undergoes alteration in structural and antigenic properties and becomes acid-labile and noninfectious. During uncoating, VP4 (a capsid protein) is released and the viral RNA is extruded from the capsid structure through the hole in the capsid caused by VP4 release into the cytoplasm. How the viral RNA gets through the endosomal membrane is not clear, but it is speculated that pore formation may occur by the interaction of the myristylated NH_2 terminus of VP4 with the endosomal membrane (Flint *et al.*, 1999). The viral RNA now becomes available for translation and replication (Fig. 9). However, only a small fraction of the viruses in the endosomes undergoes successful uncoating. The majority of the virus particles in the endosomes instead become noninfectious due to acid-induced structural alteration and are released outside the cell by the abortive pathway (Fig. 9). SV40, a naked DNA virus, also enters into the cytoplasm via receptor-mediated endocytosis. Some alteration in the SV40 virion structure occurs in the endosome as VP3, a viral capsid protein, becomes exposed. However, in the case of SV40, the virus is extruded essentially intact from the endosome into the cytoplasm and targeted to the nucleus. Therefore, the uncoating of the SV40 genome occurs in the nucleus and not in the cytoplasm. In the nucleus, the viral minichromosome (viral DNA-containing histones) is released from the capsid and becomes available for transcription and replication. Therefore, although entry of SV40 into the cell likewise occurs via an endosome, its uncoating takes place within the nucleus in a pH-independent

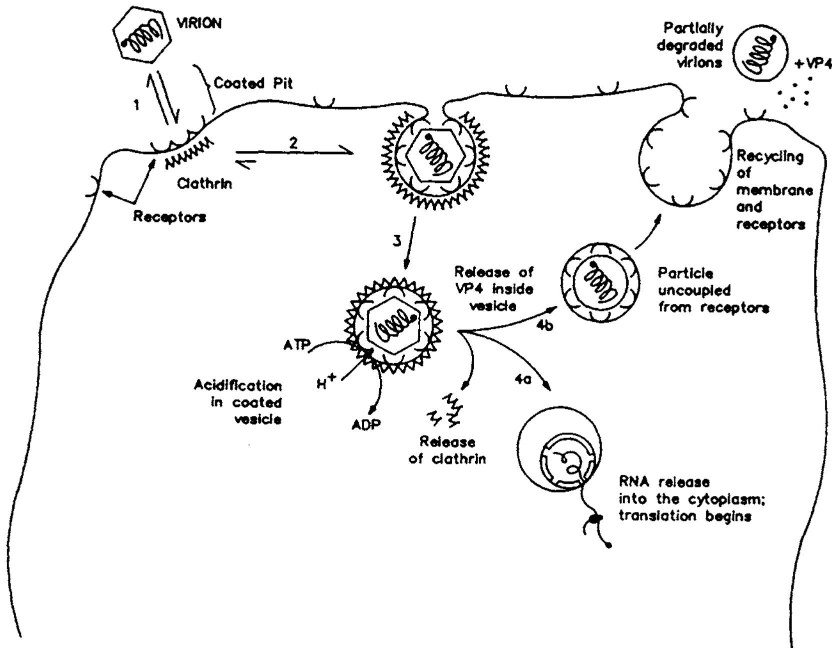


Fig. 9. Receptor-mediated endocytosis of viruses like polioviruses (steps 1 through 4a,b). The virus binds to *cell surface receptors*, usually glycoproteins, which undergo clustering at *clathrin-coated pits* (step 1) and is followed by invagination (step 2) and internalization (endocytosis) to form *clathrin*-coated vesicles (step 3). Acidification inside the coated vesicles, brought about by an energy-requiring ATPase-coupled proton pump, triggers the release of VP4 and unfolding of hydrophobic polypeptide patches previously buried inside the viral capsid. Fusion of the lipid bilayer with hydrophobic patches in the acid-unfolded capsid protein presumably triggers release and transfer of RNA from virion into the cytosol, where ribosomes can begin translating the plus-strand viral genome (step 4a). Fusion of uncoated vesicles with other kinds of intracellular lysosome-like vesicles may also be involved in the uncoating process. Some virus particles are not fully uncoated after acid-induced changes in the endosomes and are released into the extracellular medium via an abortive pathway (step 4b). These partially degraded extracellular virus particles are noninfectious. Reprinted with permission from Fields and Knipe (1990).

manner. However, how the SV40 virus is released form the endosome into the cytoplasm prior to nuclear entry remains unclear. Reovirus, a double-stranded naked RNA virus, uses the host proteolytic enzymes present in the lysosome to partially remove the outer capsid proteins and activate the core RNA transcriptase for initiation of viral mRNA synthesis.

For enveloped viruses, uncoating occurs through fusion of the viral membrane with the cellular membrane using either pH-independent or pH-dependent pathways. In the pH independent pathway, virus penetration and uncoating occur simultaneously and at the cell surface after virus–host interaction. This is best illustrated by the entry process of paramyxoviruses and retroviruses (e.g., HIV). In both cases, viruses bind to the cell surface receptors (i.e., sialic acid present either on the cell surface glycolipids or glycoproteins for paramyxoviruses and the receptor protein CD4 and coreceptors for HIV). Either one (gp160 for HIV) or two (F and HN for paramyxovirus) separate viral glycoproteins are involved in this binding and fusion processes. One of these proteins must be cleaved in the infecting virus for fusion to occur (examples being gp160 → gp120 and gp 41 for HIV and F → F1 and F2 for Sendai virus). For HIV, the gp120/gp41 complex undergoes conformational changes after binding to the cellular receptor and coreceptor, releasing the hydrophobic domain of gp41, which then functions as a fusion peptide and causes fusion of the viral membrane with the plasma membrane, thereby releasing the nucleocapsid containing the viral RNA and reverse transcriptase into the cytoplasm. Subsequently, cyclophilin A, present in HIV particles, aids in the uncoating process by destabilizing the capsid and initiating reverse transcription of the viral RNA. For paramyxoviruses, HN protein binds to the sialic acid on the cell surface receptor and induces, in some way, conformational changes in the other viral envelope protein, known as the F1/F2 complex, and thereby facilitates the fusion domain of F1 to cause fusion between the viral membrane and the plasma membrane, and release of the viral nucleocapsid containing the transcriptase (RDRP) into the cytoplasm. For paramyxovirus, the entire viral replication process takes place in the cytoplasm, whereas for retroviruses the proviral DNA is formed in the cytoplasm after reverse transcription of the viral RNA and is then transported into the nucleus for integration and transcription. How the receptor–protein interaction facilitates conformational changes leading to fusion of the viral and cellular membranes in a pH independent manner is not fully understood. Furthermore, fusion for these viruses occurs not only between viruses and host cells but also between virus-infected cells expressing the cleaved viral membrane proteins on the cell surface and uninfected cells containing the receptors (and coreceptors) present on the cell surface. These cell-to-cell interactions lead to formation of syncytium or multinucleated giant cells. Such multinucleated giant cells are important diagnostic markers for a number of viral infections (e.g., respiratory syncytial virus [RSV], mumps, measles viruses). The process of fusion of HIV-infected cells to uninfected $CD4^+$ T cells is implicated in AIDS pathogenesis, which causes depletion of $CD4^+$ T cells in HIV infected persons.

For other enveloped viruses like VSV and influenza viruses, penetration and uncoating are two separate events. Following receptor binding, these other viruses enter the cytoplasm by receptor-mediated endocytosis, and fusion and uncoating

occur within the endosome in a pH-dependent (low pH of ~5) manner. The fusion and uncoating of these viruses can be blocked by agents like monensin, which increases endosomal pH. For VSV, the G protein binds to the receptor and becomes activated for fusion at low pH, even though it remains uncleaved. Although the VSV G protein contains a hydrophobic fusion region, the mechanism of its fusion process within the endosome is not well understood. The fusion and uncoating processes are best understood at the molecular level for influenza viruses. Again, for influenza viruses, although fusion and uncoating occur simultaneously, they are considered two separate events. Following binding to sialic acid on the cell surface receptor, influenza virus undergoes receptor-mediated endocytosis and the cleaved HA trimer (i.e., HA1/HA2 heterotrimer complex) present on the viral membrane undergoes conformational changes at the low pH of endosomes (~5).

Acidic pH specifically alters the structure of HA2, which attains the fusiogenic state. In conjunction with this process, HA1 becomes dissociated from the stem of the HA spike, and the fusion peptide present at the NH_2 terminus of HA2, which normally remains buried in the protein interior of the HA trimer, is released and the polypeptide structural loop becomes transformed into a helix to form an extended coiled coil structure that relocates the hydrophobic fusion peptide toward and into the target (endosomal) membrane (Fig. 10). This process leads first to hemifusion by mixing of the outer lipids of the bilayers of both viral and endosomal membranes and later to complete fusion of both lipid bilayers of the membranes, leading to formation of a pore between the two compartments. Subsequently, the pore dilates and leads to mixing of the cytosol and virion contents and delivery of the viral nucleocapsid into the cytoplasm (Fig. 11). In addition to causing fusion, low pH also aids in the uncoating of influenza viral nucleocapsid. Uncoating in this case is defined as the separation of a nucleocapsid from the virus matrix protein (M1). Therefore, with this type of virus, low pH (~5) is not only crucial to the outside of the virus particle (virion) for inducing conformational changes of HA1 and HA2 but is also needed inside the virus particle for separation of M1 from the nucleocapsid. The viral membrane also possesses a small number of M2 tetramers (16 to 20 per virus particle) formed by a small transmembrane type III M2 protein. These M2 tetramers are ion channels that remain closed at neutral pH and open at low pH (~5) to allow protons (H^+) to enter from the endosomes into the core of the virus particle. The resulting acidic pH inside the virus particles causes dissociation of M1 from the viral RNP (also known as the vRNP or nucleocapsid) containing vRNA, and so the M1-free viral RNP is released into the cytoplasm (Fig. 12). Both the opening of the M2 ion channel and the uncoating of some influenza A viruses can be blocked by amantadine (or rimantadine), a drug currently used in treating influenza infection. The dissociation of M1 from the vRNP is important since the released vRNP can now be

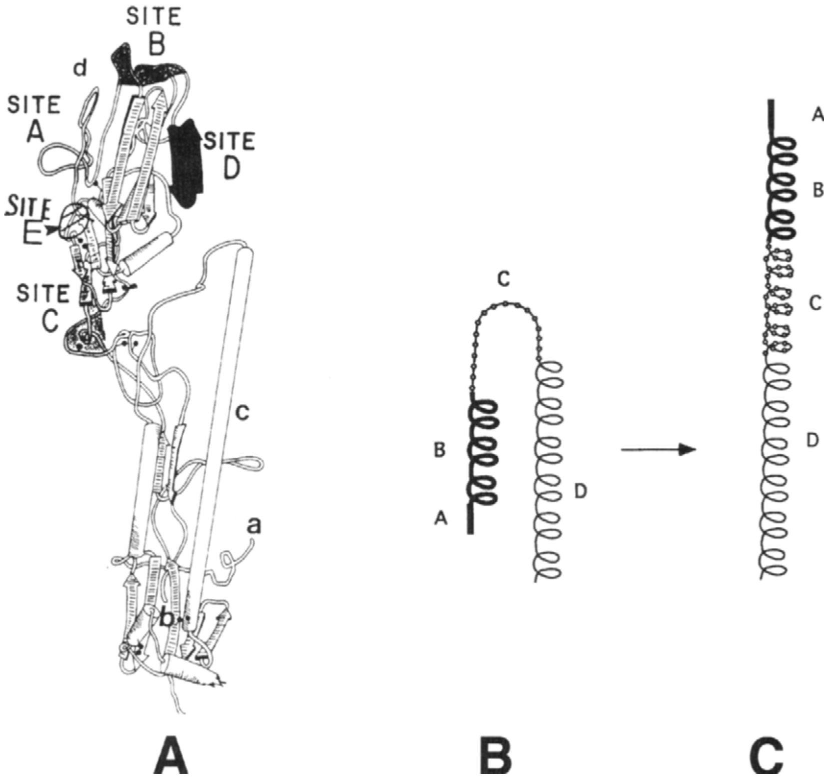


Fig. 10. A model for the fusiogenic state of HA of influenza virus. HA in its native state is a trimer. However, for demonstration of the conformational changes during fusion, only a monomer is depicted in this figure. Panel **A** shows an HA monomer in its native state, containing the cleaved HA1 and HA2. Five epitope sites (A–E) are shown on the globular head. "a" and "b," respectively, are the NH_2 terminus of HA2 and the COOH terminus of HA1 after cleavage. Panel **B** shows the native form of HA2 (again only a monomer is depicted) in which the NH_2 terminus (A) would be buried inside the core of the coiled coil of HA trimer. B and D represent two α helices, and C is the loop region. Panel **C** shows the fusiogenic state at pH 5, at which point the HA1 subunits (not shown) are dissociated from the stem and the fusion peptide (A) at the NH_2 terminus of HA2 is released from the protein interior, and the loop (C) has "sprung" into a helical conformation to form an extended coil that relocates the fusion peptide (A) 100 Å toward the target membrane to promote membrane fusion. Note the conversion of the native form of HA2 in panel **B** to the fusiogenic state in panel **C**.

translocated into the host nucleus where the transcription and replication of vRNA can occur. M1, on the other hand, interferes with transport of vRNP into the nucleus and also inhibits vRNP transcription.

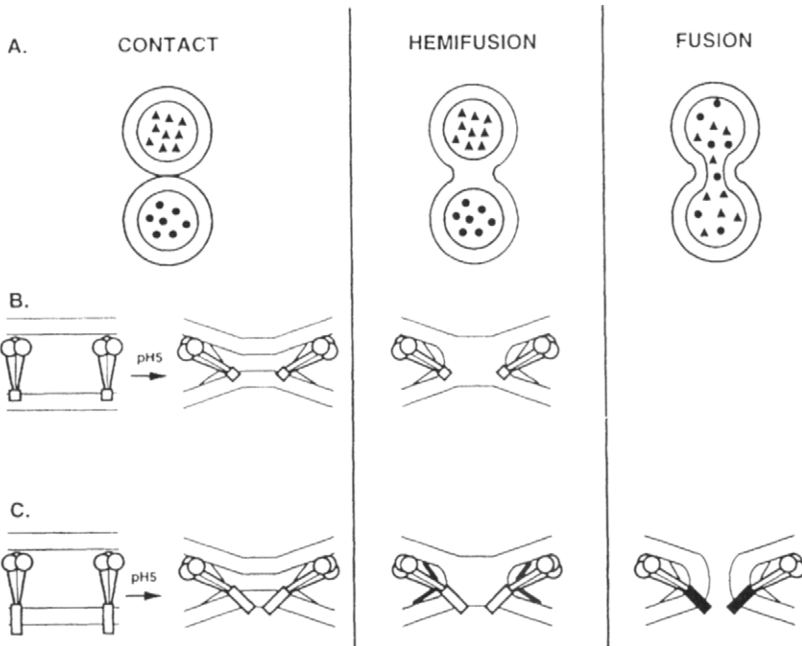


Fig. 11. Membrane fusion via a hemifusion intermediate. (**A**) Hemifusion in model systems. Two membrane-bound vesicles contact (CONTACT). Next, they form a hemifusion intermediate (HEMIFUSION). In this state, lipids of the outer leaflets, but not the inner leaflets, mix. At the point of hemifusion, the aqueous contents of the two vesicles (triangles and circles, representing host cell and virion) still remain separated. Next, the lipids of the inner leaflets mix and complete the fusion process (FUSION), resulting in mixing of the aqueous contents of both vesicles, that is, releasing the viral nucleocapsid into the cytoplasm of the infected cell. (**B**) and (**C**) represent enlargements of the respective contact areas from **A**, showing the proposed placement of HA (hemagglutinin protein) trimmers. After opening of the initial narrow fusion pore, the pore dilates (not shown). Reprinted with permission from Kemble *et al.* (1994).

C. Targeting Viral Nucleocapsids to the Replication Site

Viral replication occurs either in the nucleus or in the cytoplasm of infected cells. For those viruses that replicate in the cytoplasm, which customarily are those with RNA genomes, except for the DNA-containing poxviruses, the uncoating process releases the viral nucleocapsid directly into the cytoplasm, which is the site of transcription and replication. For viruses that replicate within the nucleus, which tend to be the ones having DNA genomes with notable exceptions such as the RNA-containing influenza viruses and retroviruses, the viral nucleocapsids

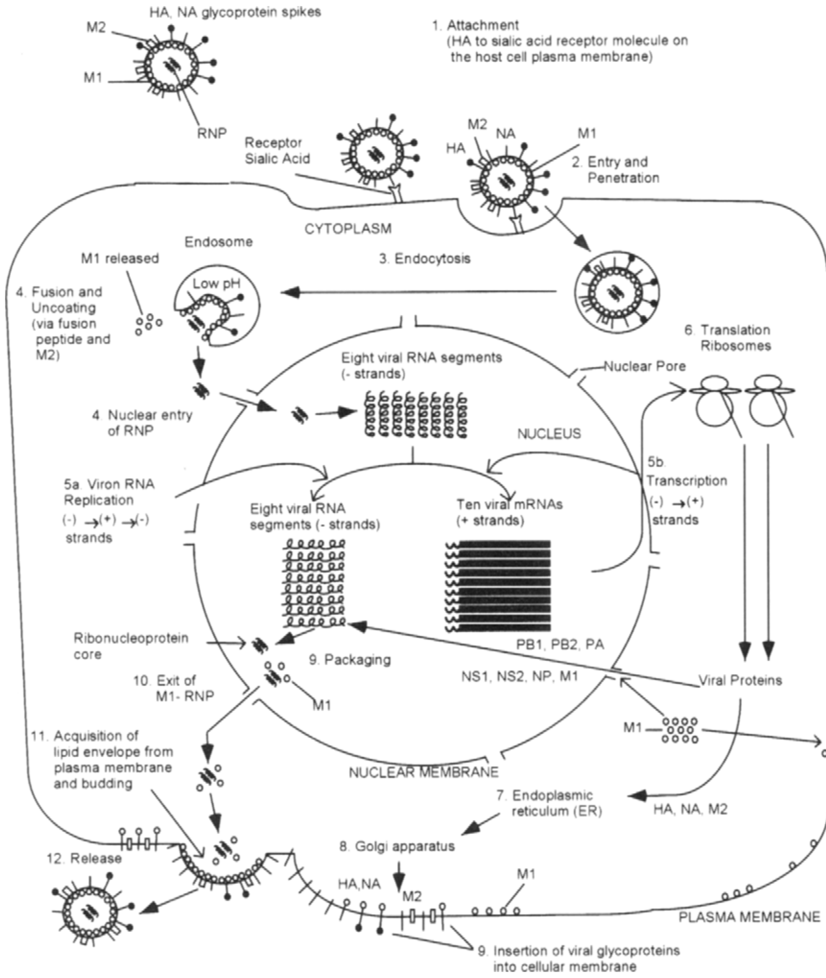


Fig. 12. Schematic presentation of the infectious cycle of an influenza virus. The steps in the replication cycle are noted as 1 (attachment) through 12 (release). PB1, PB2, PA, NS1, NS2, NP, M1, HA, NA, and M2 are the virus-encoded proteins translated from 10 mRNAs, which are transcribed from eight vRNA segments of negative polarity.

that are released in the cytoplasm after uncoating must be targeted into the nucleus. Nuclear targeting requires that these viral nucleocapsids contain proteins possessing nuclear targeting signal(s) (NTSs or NLSs), which are recognized by the cellular nuclear targeting machinery and translocated into the nucleus via nuclear pores. However, the stage of uncoating at which nuclear targeting takes place varies with viruses. For SV40, essentially the entire virus particle that has been taken into the cytoplasm is transported into the nucleus, and it is only in the nucleus that uncoating of the capsid occurs concomitant with release of the viral minichromosome. For adenoviruses, uncoating occurs at the nuclear pore where the viral nucleocapsid docks and the viral DNA is delivered into the nucleus through the nuclear pore. For influenza viruses, uncoating occurs during introduction of the nucleocapsid into the cytoplasm by dissociation of M1 from the vRNP. This M1-free vRNP is then transported into the nucleus. For retroviruses, not only uncoating but also additional biosynthetic processes — including reverse transcription of the RNA genome and synthesis of the double-stranded proviral DNA — occur in the cytoplasm. Then the retroviral DNA along with integrase is translocated into the nucleus for integration of the proviral DNA into the host genome. Transcription of the retroviral genomic and subgenomic mRNAs occurs only from the integrated proviral DNA in the nucleus. For hepatitis B virus, the partially double-stranded DNA, the viral genome following uncoating in the cytoplasm, becomes fully double-stranded and circularized in the cytoplasm, and then it is translocated into the nucleus for subsequent transcription of genomic and subgenomic mRNAs.

D. Post-Uncoating Events

The "immediate events" in the viral replication cycle, those that occur following uncoating, vary with the nature of the viral genome. For plus-strand RNA viruses except retroviruses, translation of the viral RNA follows immediately after uncoating. The viral RNA extruded from the capsid is then used by the host translation machinery for directing protein synthesis (Fig. 9). For all other viruses, whether of DNA or RNA genome, the step immediately following uncoating is either transcription of the genome yielding functional mRNAs or reverse transcription of vRNA yielding proviral DNA (retroviruses).

E. Transcription of Viral Genes

From the transcription viewpoint, viruses can be classified into two major categories, that is, whether they possess a DNA genome or an RNA genome. Of the first group, the DNA genome of different viruses varies greatly in complexity between virus families, encoding from only 4 to 5 genes to more than 200 genes

or open reading frames (ORFs). DNA viruses use DNA-dependent RNA polymerase, which can be either virus-specified (e.g., poxviral RNA polymerase) or host-specified (e.g., RNA *pol* II) to generate their mRNAs. RNA viruses, however, must use RNA-dependent RNA polymerase (RDRP), which is always virus-specified and is therefore different and specific for each virus group to generate their mRNAs.

1. Transcription of DNA Viruses

All DNA viruses except for the poxviruses transcribe and replicate their genomic material in the host cell nucleus. Poxviruses transcribe and replicate in the cytoplasm. In addition, all DNA viruses except poxviruses use host *pol* II for transcription of their DNA into mRNAs. Poxviruses use virus-specified polymerase for transcription of its genome. Viral DNA genomes like host DNA often possess the *cis*-acting elements, which are essential for successful transcription of their DNA. These elements are called the viral promoter and enhancer. The promoter is the RNA polymerase binding site on viral DNA (e.g., TATA box, CAT box, GC box) localized in the vicinity (usually upstream) of the transcription initiation point. The enhancer element, which enhances transcription of the viral mRNA over the basal level, is found either in the proximal or distal region of the promoter and may be located either upstream or downstream of the promoter element. Transcription of the viral DNA genome can broadly be divided into the early and late phases. Early genes are usually catalytic and regulatory in nature, involved in regulating transcription of mRNAs and replication of viral DNA. Late genes usually produce mRNAs for structural viral proteins, which are the major components of either the viral capsid or envelope. Early genes are usually transcribed prior to initiation of viral DNA synthesis, and late genes are transcribed only after viral DNA synthesis has been initiated. Thus, synthesis of the progeny viral DNA demarcates the dividing line between early and late gene transcription. However, for complex viruses such as herpes simplex virus (HSV), the different classes of regulatory genes, for example, immediate early (α), delayed early (β), and late (γ1, γ2) are transcribed at different phases of the viral replication cycle, each having different regulatory functions for either turning on or shutting off other viral genes. Viral genes can be transcribed from either of the two DNA strands, with the coding sequences thus running in a direction opposite to a duplex DNA. These viral genes usually possess the structural features of eukaryotic cellular genes, and the viral mRNAs similarly undergo posttranscriptional processing like cellular genes. The mRNAs are usually capped at the 5′ end, polyadenylated at the 3′ end, and may undergo posttranscriptional splicing prior to their exit from the host cell nucleus. However, poxviral mRNAs, which are also capped and polyadenylated, do not undergo splicing since they are made in the cytoplasm.

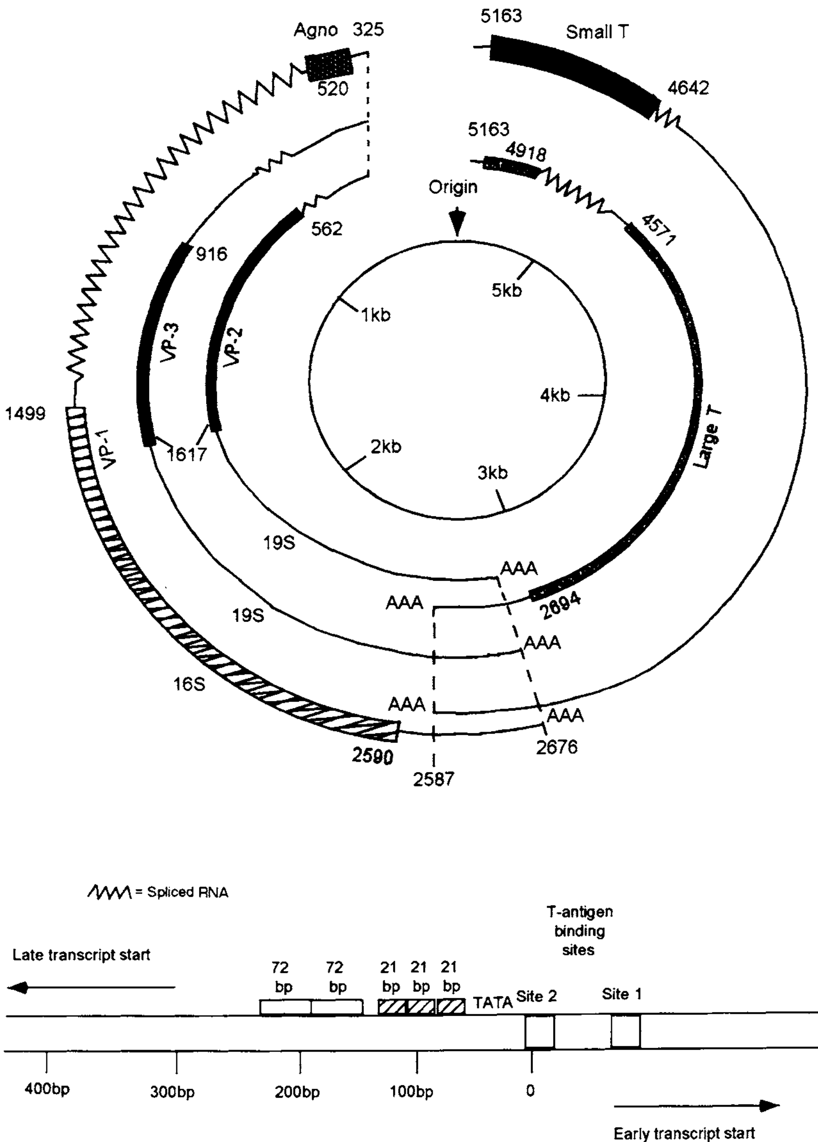


Fig. 13. Genome and transcription map of SV40 (top). The origin of replication is shown at the top of the inner circle. The numbers indicate the nucleotide position in the SV40 DNA, while zigzag markings indicate spliced introns. Different shaded regions indicate different protein-coding sequences. The bottom drawing shows the details of the transcription regulatory elements in the proximity of the "origin" region and the direction of the early and late transcription.

An example of transcription of a small double-stranded viral DNA genome (SV40) is shown in Figure 13. Transcription of the SV40 genome is carried out by the host cell's RNA polymerase II. Early mRNAs (large T and small T) are transcribed from the early promoter of the early DNA strand, whereas late mRNAs (i.e., the mRNAs for VP1, VP2, VP3 and agno proteins) are transcribed from the late promoter and the opposite DNA strand. Both early and late transcription in SV40 are initiated from the common control region in opposite directions at different phases of the replication cycle. This control region also regulates viral DNA replication. This region consists of a series of repeat elements with different functions: three 21-base repeats that together contain six copies of GC-containing hexamers and serve as the promoter for early transcription. Downstream of these repeats is a TATA-box and upstream are two 72-base repeats constituting the enhancer element (Fig. 13, bottom). These three regulatory elements bind specific cellular factors and are important in regulating early transcription. Of these, the 21-bp repeats and the enhancer elements are also important in regulating late transcription. The switch from early to late transcription is brought about by binding of large T antigen to specific sites in the control region and a change in the replicative state of the viral DNA. Large T (LT) and small T (ST) antigens are two early proteins, translated from two different mRNAs produced by differential splicing. The two late mRNAs have a common untranslated region and a common poly(A) addition site but are generated by differential splicing. Each of these late SV40 mRNAs are bicistronic, with alternative initiation codons. One of these late mRNAs is translated into VP2 and VP3, and the other into VP1 and the agno protein.

On entry into the cytoplasm of the infected cell, hepatitis B virus (HBV), a partially double-stranded DNA virus, uses virus-specified reverse transcriptase (P) to synthesize the complete circular DNA, which is then transported into the nucleus. Host cell *pol* II subsequently transcribes its genomic and subgenomic mRNAs from different initiation points (Fig. 14). They are all capped at the 5′ end, unspliced, and have a common termination and poly(A) addition site at the 3′ end. Different classes of genomic-length (3.5-kb) RNAs, possessing different 5′ but common 3′ termini, function as a template for making cDNA or are translated into the Pre-C and C proteins as well as the P protein. Subgenomic mRNAs are translated into the Pre-S1, Pre-S2, and S proteins as well as the X protein (Fig. 14).

2. *Transcription of RNA Viruses*

Among the different families of RNA viruses, the RNA viral genome appears to be much less complex as compared to the genomes of the highly complex DNA viruses. However, these RNA viruses use multiple strategies to encode different mRNAs and different proteins. Unlike DNA viruses, the majority of the RNA viruses (except for retro-, orthomyxo-, and related viruses) replicate in the cyto-

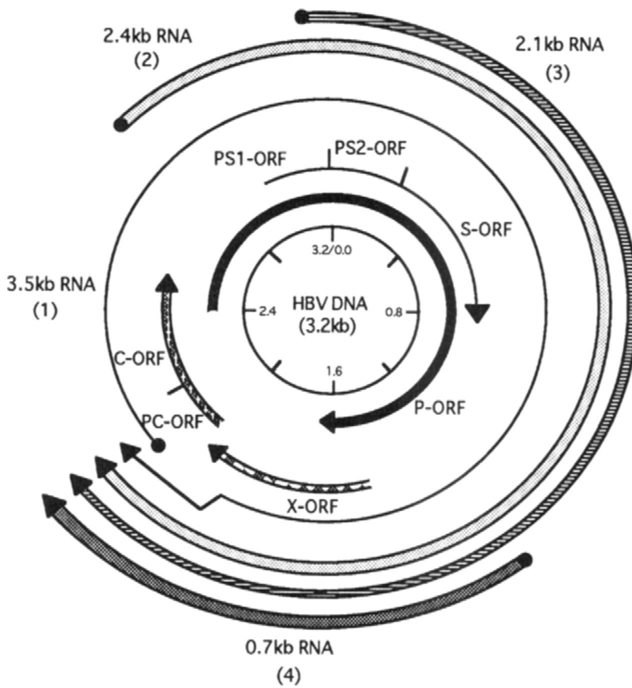


Fig. 14. Replication, transcription, and translation of hepatitis B virus (HBV) DNA. Four RNA classes: 3.5 kb (1), 2.4 kb (2), 2.1 kb (3), and 0.7 kb (4) are transcribed. The 3.5-kb product (#1) is used for full-length DNA (minus-strand) synthesis. Different classes of 3.5-kb product also function as mRNAs whose translation products are HBcAg, the polypeptide consisting of the PC-ORF (pre-core), and C-ORF (core) and P-ORF (P-protein, also called either polymerase or reverse transcriptase). The 2.4-kb mRNA (#2) makes a large protein consisting of the polypeptides PS1-ORF, PS2-ORF (presurface), and S-ORF (surface protein). The 2.1-kb mRNA (#3) makes the S-ORF (surface) protein, and the 0.7-kb mRNA (#4) encodes the X-ORF protein.

plasm, so that their mRNAs cannot undergo RNA splicing. RNA viruses also possess genes for regulatory and catalytic proteins as well as for structural proteins. However, transcription of mRNAs encoding these proteins is not as strictly demarcated with respect to the timing of their genomic nucleic acid replication, as is found for DNA viruses. On the other hand, with RNA viruses there is a great deal of variation in the level of transcription of different viral mRNAs. The mRNAs of the major structural proteins — like the nucleoprotein (NP) and matrix (M) glycoproteins — are usually made in larger amounts as compared to the lower amount of mRNAs synthesized for catalytic (e.g., polymerases) proteins. For unsegmented negative-strand RNA viruses, the level of mRNA transcription is regulated by the promoter-proximal position of a gene (e.g., for VSV or

paramyxoviruses, see Fig. 3) or by an internal promoter that produces subgenomic mRNAs (e.g., togaviruses). The strategy used by different RNA viruses for mRNA transcription depends on the nature of the RNA genome (+ or – strand, segmented or unsegmented) and whether the nucleocapsid is icosahedral or helical.

a. For plus-strand icosahedral naked RNA viruses (e.g., poliovirus), the entire viral genomic RNA functions as the only mRNA and is translated from one ORF into a large polyprotein, which is then cleaved by specific proteases into different functional proteins representing the RNA polymerase and the capsid proteins (VP1, VP2, VP3, VP4), and so on (Fig. 2).

b. For some enveloped plus-strand RNA viruses (e.g., togaviruses), the 5′ half of the viral genomic RNA encodes and is translated into nonstructural (catalytic) proteins involved in RNA transcription and RNA replication, whereas a separate subgenomic 26-S mRNA (+), made from an internal promoter on the minus-strand RNA template, encodes the structural proteins (i.e., capsid and envelope proteins). This 26-S mRNA is synthesized in a larger quantity than is the genomic length RNA. However, another group (flaviviruses) of enveloped plus-strand RNA viruses possesses one large ORF in its genomic RNA encoding a single large polyprotein, which, as is the case with picornaviruses, is cleaved into specific proteins by a virus-encoded proteinases.

c. For coronaviruses, which contain a large plus-strand RNA genome of ~30 kb, multiple subgenomic mRNAs are found. However, each of these mRNAs possesses the same 5′ leader (i.e., leader-primed transcription) and the common 3′ end containing poly(A) sequences. These mRNAs therefore contain the nucleotide sequence of more than one ORF. Usually, however, only the first ORF at the 5′ end of mRNA is translated into protein.

d. Minus-strand RNA (-) viruses replicating in the cytoplasm may possess either one large genomic RNA molecule (unsegmented) or two or more different subgenomic RNAs (segmented). For those viruses that possess an unsegmented genomic RNA molecule (e.g., VSV), the viral genes are arranged sequentially in the genomic RNA (–) with stop, intergenic, and start (EIS) sequences (Fig. 3). RNA polymerase (RDRP) synthesizes the virus mRNAs by initiating transcription at the 3′ end (one entry) and then terminates at the stop sequence (E) of that gene, skips the intergenic sequence (I), and initiates at the start (S) sequence of the next gene, and so on. Therefore, the RNA polymerase sequentially transcribes the downstream genes and there is no independent internal entry of the RNA polymerase on the genome. Since RNA polymerase randomly falls off during transcription and cannot initiate internally, the mRNA level (and, consequently, the protein level) is determined by the location of a particular gene in the viral genome. For example, mRNA of the capsid protein (N or NP) is present at the extreme 3′ end of the minus-strand (i.e., proximal to the promoter) just after the leader (ℓ) sequence, and it is therefore made in the most abundant amount, since it is the first gene to be transcribed by RDRP into mRNA. On the other hand, the L (polym-

erase) gene, encompassing nearly half of the genome, is located at the 5′ end of the viral RNA (distal to the promoter), so that the L mRNAs and L proteins are made in the least amount (Fig. 3). Each mRNA is capped at the 5′ end and polyadenylated at the 3′ end by the virally encoded RDRP.

e. Orthomyxoviruses, which are segmented, minus-strand RNA viruses, possess 8 RNA segments, which in total encode 10 mRNAs and 10 proteins for type A and B viruses. Orthomyxoviruses are transcribed and replicated in the nucleus. Orthomyxoviruses use a unique strategy to initiate transcription. They cannot initiate *de novo* mRNA transcription without a primer and must use the host's capped RNA as the primer at the 5′ end for mRNA transcription. One of the three proteins (PB2) of the viral polymerase complex (PB1/PB2/PA) recognizes the newly synthesized capped host RNA and cleaves the capped host RNA around 12–15 nucleotides from its 5′ end. Then another protein (PB1) of the polymerase complex uses the capped primer for viral mRNA initiation and chain elongation. Therefore, each influenza viral mRNA possesses at its 5′ end a capped nonviral RNA sequence acquired from the host nuclear RNA (Fig. 15). In addition, two viral RNA segments (segments #7 and #8) generate both unspliced and spliced mRNAs, causing translational shift to a different reading frame. In this process, 8 influenza viral RNA segments of type A and B viruses give rise to 10 mRNAs and 10 proteins (Nayak, 1997).

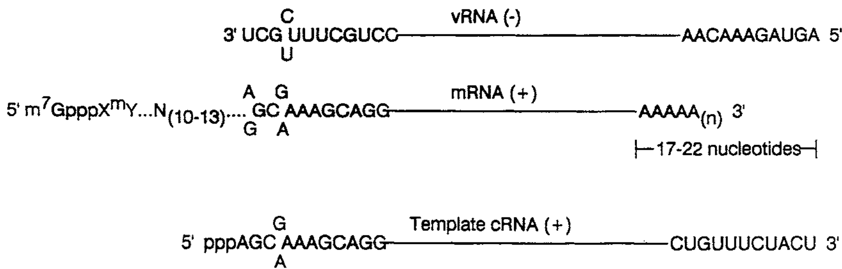
Segmented ambisense RNA viruses (e.g., arenaviruses) on infection produce a subgenomic mRNA using the 3′ end of the genomic RNA as the template, and later on in the infectious cycle use the antigenomic RNA as the template to generate the mRNA with the same polarity as the 5′ end of genomic RNA.

f. Viruses that possess double-stranded (ds) RNA viral genomes, such as reoviruses, are segmented and replicate in the cytoplasm. Their viral transcriptase, which is also present within the virus particles, synthesizes single monocistronic mRNAs from each dsRNA segment.

g. Retroviruses, although possessing a plus-strand RNA genome, contain reverse transcriptase (RT) in the virion. Transcription of retroviral mRNAs occurs in the nucleus from the integrated proviral DNA template by the host RNA *pol* II. Usually, both the unspliced genomic-length mRNA as well as the subgenomic mRNA, the latter being produced by splicing in the nucleus, function in protein translation.

F. Translation

Virions have evolved to become very efficient organisms that package a relatively small amount of genomic DNA or RNA in their capsids but use this information efficiently to generate the maximum number of functional proteins



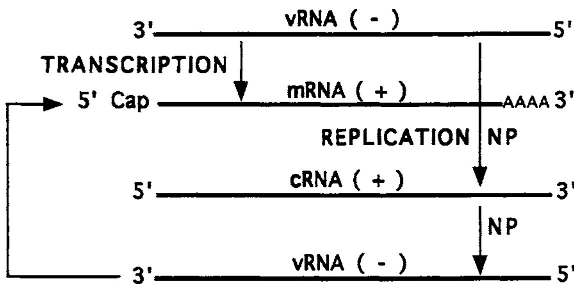


Fig. 15. Transcription and replication of the influenza virus RNA (vRNA). (**A**) The three classes of influenza virus-specific RNAs found in the virus-infected cells, vRNA of minus (–) polarity, and cRNA and mRNA, both of (+) polarity. Note that the viral mRNA (+) possesses nonviral (host) capped sequences at the 5′ end and lacks sequences of 17–22 nucleotides from the 3′ end but contains poly(A) sequences. The template cRNA (+ strand), on the other hand, is an exact copy from end to end of the vRNA (– strand) and does not possess either cap at the 5′ end or poly(A) at the 3′ end. (**B**) The transcriptive and replicative processes of influenza viral RNA.

required to produce infectious progeny virions. For some viruses like VSV, all of the viral proteins encoded by the genome and produced in the infected cells including the transcriptase are incorporated into the virion and become structural components of virus particles. For these viruses, there are by definition no non-structural proteins, that is, there are no proteins that are encoded in the virion genome and produced in the infected cells but not incorporated into the virion. However, for the majority of viruses, one or more nonstructural proteins, either catalytic (enzymatic) or regulatory, are synthesized in virus-infected cells. These nonstructural proteins are required for the infectious cycle but are not incorporated

into virion particles. Both structural and nonstructural proteins are translated from viral mRNAs, and the majority of viral mRNAs (except in the case of picornaviruses) possess structural features similar to that of the host mRNA (i.e., they possess a cap at the 5′ end, a translation initiation triplet [AUG] in the context of Kozak's rule, as well as translation termination triplets and poly(A) sequences at the 3′ end). These viral mRNAs undergo cap-dependent ribosome binding and ribosome scanning to locate the proper initiation triplet, a process that does not provide any advantage over the host mRNAs during translation. Therefore, after infection, the virus must overcome two major problems to achieve successful replication: (1) viruses must somehow overcome competition from host mRNAs for using translation machineries, and (2) viruses that possess only a limited amount of coding information must still be able to generate the considerable number of functional proteins needed for replication.

1. Viruses have developed a number of strategies to compete with host mRNAs for efficiently using the host translation machinery. These include the following. (a) Viral transcription machinery (especially in RNA viruses) are more efficient in generating high levels of mRNAs so that they can outcompete host mRNAs in translation. (b) Some viral proteins target and interfere with the host transcription machinery so that the host transcription level goes down or shuts off. Influenza viruses, however, use a novel system to their advantage. As mentioned earlier, one of the influenza polymerase proteins, PB2, recognizes, binds to, and cleaves the newly synthesized capped host hnRNAs around 13–15 nucleotides, and the capped oligonucleotide is used as the primer for mRNA synthesis. The cleavage of host hnRNAs, in turn, prevents host mRNA synthesis and processing. In addition, this virus interferes with nuclear export of the host mRNAs. (c) Some viruses modify the host translation machinery to use that machinery for its advantage while simultaneously shutting off host mRNA translation. This latter mechanistic approach is particularly evident for picornaviruses, which inactivate the cap-binding protein and modify the host translational factors (e.g., eIF2, eIF3/4B) and thus shut off cap-dependent host mRNA translation. However, picornaviral mRNA can still be translated efficiently because it does not have a cap at the 5′ end but rather possesses a unique RNA secondary structure known as an internal ribosome entry site (IRES) and is independent of Kozak's rule. The picornaviral mRNAs possessing an IRES can be translated efficiently in a cap-independent manner, while capped host mRNAs cannot be translated because of viral-mediated inactivation of some of the host translational factors.

2. Viruses have developed different strategies to produce a relatively large number of functional proteins from a small amount of genetic information using both transcriptional (or posttranscriptional) as well as translational (or posttranslational) processing.

a. Transcriptional (or posttranscriptional) generation of different mRNAs. Double-stranded DNA viruses can use both of their DNA strands to transcribe mRNAs, thereby increasing potential transfer of information into proteins. Some viruses that make mRNAs in the nucleus (either RNA or DNA viruses) can generate different mRNAs from the same genomic strand by using either unspliced mRNA or electing alternative splicing sites, thus even causing frame shifts in the subsequent translation. Influenza viral proteins M1, M2, NS1, NS2, and SV40 proteins (such as VP1, VP2, and large T and small T antigens) are classic examples of generating different mRNAs and proteins through splicing. Some viruses use RNA editing (i.e., nontemplated nucleotide addition in the mRNA) to shift the translation frame. This latter technique is frequently used by paramyxoviruses to generate their V and C proteins. Hepatitis delta virus uses adenosine deaminase for RNA editing as part of the transcription process to generate its δ Ag-L antigen. Other viruses selectively use different promoters to generate genomic and subgenomic mRNAs (e.g., HBV, togaviruses).

b. Translational (and posttranslational) generation of different viral proteins. The most common way to generate a number of functional proteins after translation is by proteolytic cleavage. These endoproteases, usually encoded by the virus, are sequence-specific and can generate a number of functional proteins from one large viral polypeptide. Classic examples of this type of cleavage activity are found with poliovirus (picornavirus) and flavivirus proteins. Poliovirus RNA is translated into a large polypeptide that sequentially undergoes endoproteolytic cleavage by different poliovirus proteases at specific amino-acid sites, generating 11 viral proteins (VP4, VP2, VP3, VP1, 2A, 2B, 2C, 3A, VPg, 3C, 3D) and other intermediate proteins (Fig. 2). The importance of virus-specific proteases was recently demonstrated in HIV infection, during which HIV protease inhibitors alone or in combination with RT (reverse transcriptase) inhibitors can be used in the treatment of AIDS to reduce patient virus load. Cleavage by host proteases is also sometimes critical to render viral proteins functional and viral particles infectious (e.g., conversion of influenza viral HA to HA1 and HA2, HIV gp160 to gp120 and gp 41).

Different initiation codons are also used in bicistronic mRNAs to translate different proteins. Depending on the initiation codon used, either one or the other protein can be translated (e.g., NB protein and NA protein from the same mRNA in influenza virus type B). Usually, one of the initiation codons is favored, thus regulating the levels of the two proteins produced from one bicistronic messenger RNA. Another strategy, often used by retroviruses, is either translational frame-shift or translational suppression of termination codons. Translational frame-shift due to ribosomal slippage causes generation of the *gag*–pro–*pol* fusion protein in avian leukosis virus. This protein is then cleaved by a virus-specific protease (usually aspartic proteases) to generate individual functional proteins. Similarly,

some retroviruses use translational termination suppression to continue translation in the same reading frame. In the *gag*–UAG–*pol* sequence, translation is normally terminated after the *gag* protein at the UAG codon. Occasionally, termination at UAG can be suppressed by a minor host tRNA capable of inserting glutamine and thereby generating a *gag–pol* fusion protein, which subsequently is cleaved by a viral protease to generate *gag* and *pol* proteins. Again, both frame-shift and/or in-frame suppression produces only a minority of fusion proteins with *pol*, thus regulating the amount of *pol* protein needed in small amounts in virus-infected cells.

G. Replication of Viral Genome

The replication pathway of different viral genomes varies depending on the nature of the viral genome. The overall strategy of viral genome replication can be grouped into seven pathways depending on the nature of the genome (Fig. 16). While all DNA viruses of eukaryotes except poxviruses replicate in the nucleus of their host cells, some use cellular DNA polymerase and others use DNA polymerase encoded by the virus genome (Table II). Poxviruses replicate their genome in the cytoplasm and use polymerase encoded by the viral genome (Table III). All RNA viruses except for retroviruses use an RNA-dependent RNA polymerase (RDRP) encoded by their own genome. Some of these (minus-stranded RNA viruses) carry RDRP in the virus particle to initiate transcription/replication of viral RNA following their entry and uncoating inside the cell. Retroviruses require reverse transcriptase (RT), an RNA-dependent DNA polymerase, in the virion particle to initiate replication. The majority of RNA viruses of eukaryotes replicate in the cytoplasm, except for the orthomyxo- and related viruses and the retroviruses. Orthomyxoviruses require cellular capped 5′ RNAs as primers for mRNA transcription, and retroviruses require production of proviral DNA and its integration into the host DNA as a prelude to both transcription and replication of the viral genome.

1. Replication of DNA Genome

Smaller DNA viruses (papova- and parvoviruses) rely on the host cell DNA polymerase, whereas more complex DNA viruses use their own virus-encoded DNA polymerase (Table II). The step for switching from transcription to replication of DNA viral genomes is primarily determined by the level of early viral proteins, which often are both regulatory and catalytic in nature. For SV40, when a sufficient amount of large T (LT) antigen has been synthesized, binding of the

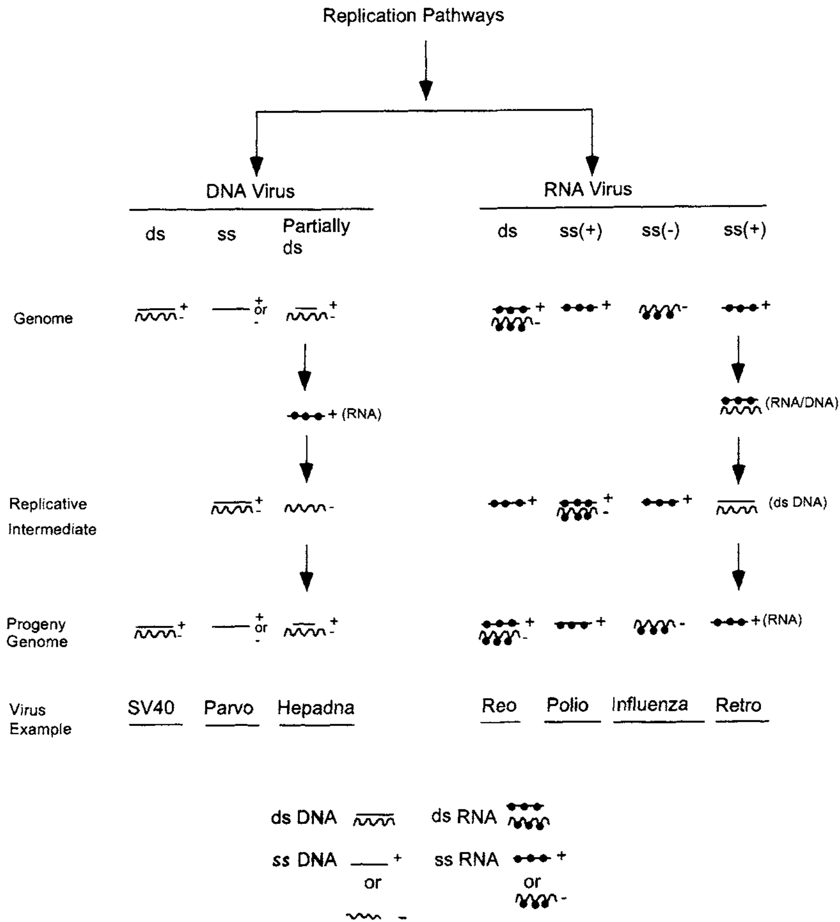


Fig. 16. Seven replication pathways of the DNA and RNA genome of viruses. Examples of different viruses with DNA or RNA genomes are indicated. ds = double-stranded; ss = single-stranded; + and – indicate positive and negative polarity.

LT antigen-initiation complex to the transcription start site of early mRNAs (Fig. 13) causes suppression of early mRNA transcription. The helicase activity of the virus LT antigen then unwinds the DNA molecule, creating a replication bubble, whereupon the host DNA primase–polymerase α complex initiates DNA synthesis using an RNA primer, creating a replication fork. Synthesis of the SV40 DNA continues bidirectionally, creating circular intermediates (Fig. 17C). Adenoviruses use asymmetric DNA-replication, which initiates DNA synthesis at the 3′ end of one strand (template strand). At the 5′ end of that strand, a 55-kD protein, covalently linked to the DNA, is needed for initiation of DNA replication. The new growing opposite DNA strand then displaces the preexisting opposite strand. The displaced strand forms a panhandle structure by pairing the inverted terminal repeats before its own replication begins (Fig. 17A). In poxvirus DNA, two complementary forms are joined at the terminal repeat sections, forming palindromes. During replication, concatamers of two genomic length strands are formed. Unit length genomic molecules are then formed by separating the staggered ends and ligation (Fig. 17E). Linear herpes virus DNA becomes circularized inside the host cell nucleus and then replicates as a rolling circle, forming tandem concatamers. Finally, the unit length genomic DNA molecules are excised from concatamers (Fig. 17B). Single-stranded parvoviral linear DNA has terminal palindromes that form hairpin structures. These hairpins then serve to covalently link the plus and minus DNA strands and self-prime the replication. The progeny viral DNA genomes are then made by strand displacement (Fig. 17D).

Hepatitis B virus DNA uses reverse transcription for replication (Fig. 17F). The partially dsDNA in the virion contains a complete minus and a partial plus strand. After infection of the cell, the virion-associated reverse transcriptase renders the partially double-stranded viral DNA into a circular duplex DNA in the cytoplasm, which is then translocated into the nucleus and transcribed into a full-length plus-strand RNA by the host RNA *pol* II that already is present in the nucleus (Fig. 14). This full-length plus-strand viral RNA is encapsidated, transported into cytoplasm, and reverse transcribed into a full-length minus-strand and a partial plus-strand DNA before being released as infectious virion.

2. *Replication of RNA Genome*

Viral RNA genomes can be single-stranded and comprised either of a plus or minus strand, or double-stranded. Furthermore, while the genomes of some RNA viruses are segmented (multiple RNA molecules), others are nonsegmented (i.e., one RNA molecule) (Tables I and III). Switching from transcription to replication in the viral infectious cycle usually occurs after sufficient amounts of the capsid protein (e.g., the nucleoprotein) have been synthesized. The nucleoprotein functions as a regulator for switching from transcription to replication of the genome.

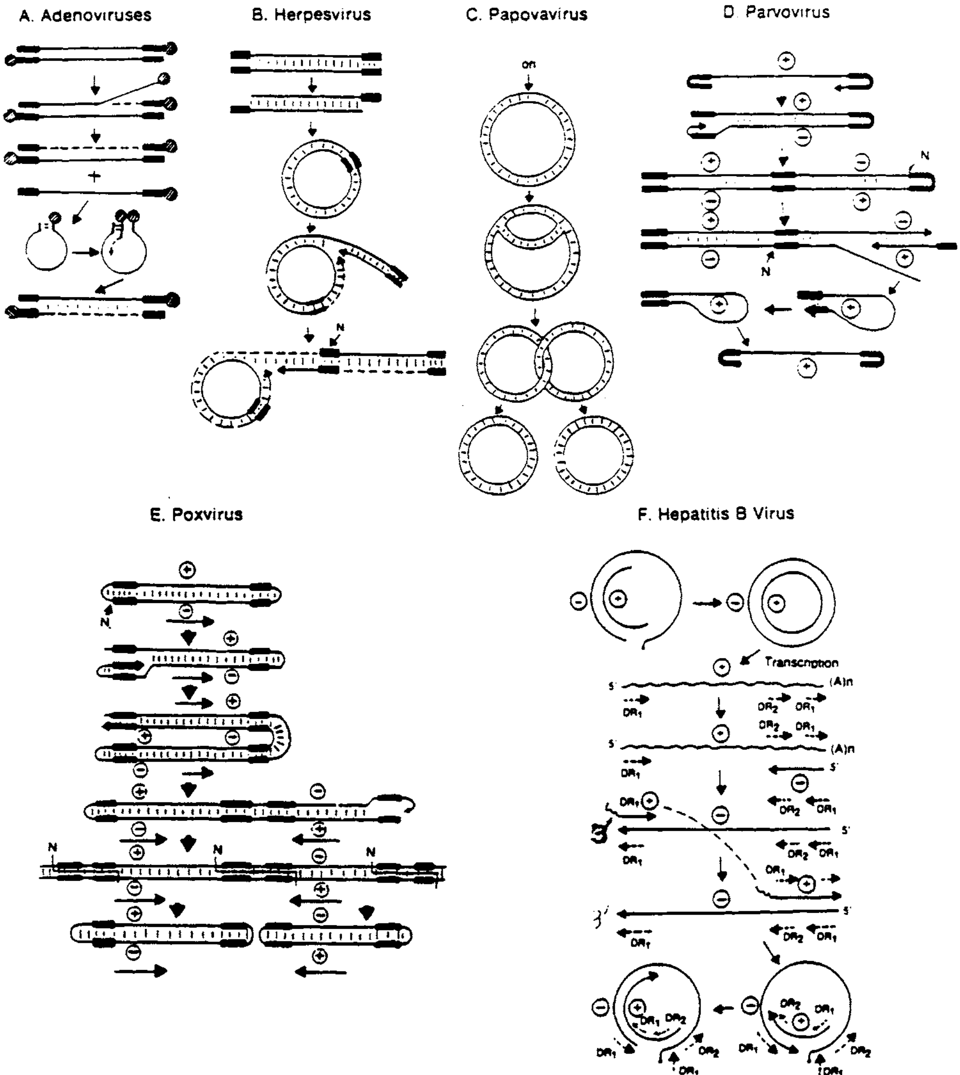


Fig. 17. Replication pathways for viral DNA genomes. In panel **A**, dashed circles are terminal viral proteins attached to the 5′ end of the DNA strands. In panels **B** and **D**, N represents endonuclease cleavage site. The heavy lines shown in panel **D** are palindromes and self-priming steps, with + and − representing strand polarity. In panel **F**, the wavy lines represent RNA and the dashed lines represent DNA coding for direct repeats DR1 and DR2. Reprinted with permission from Davis *et al.* (1990).

Inhibition of nucleoprotein or protein synthesis will also inhibit vRNA replication without necessarily interfering with mRNA synthesis. The same core enzyme (i.e., RNA-dependent RNA polymerase, [RDRP]) is used for both transcription and replication, but the enzyme (and possibly the template RNA) becomes modified by viral nucleoprotein and cellular factors to effect the switch from transcription mode to replication mode. There are five different classes of RNA genomes, based upon the different strategies that these viruses use for genome replication (Table III).

a. ***Replication of Single-Stranded Viral RNA***. Plus-strand RNA viruses are copied into a complete minus-strand RNA, which then serves as a template for synthesis of more plus strands via replicative RNA intermediates (Fig. 16). Minus-strand unsegmented RNA genomes are transcribed into two types of plus-strand RNAs: (a) subgenomic mRNAs (+ sense), which represent specific portions of the genome and are translated into proteins, and (b) full-length cRNA (+ sense), which represents a complete copy of the entire minus-strand genome and serves as the template for genomic RNA (– sense) synthesis. The synthesis of cRNA is regulated by a switch from transcription to replication that occurs after sufficient amounts of capsid proteins (e.g., NPs) have been synthesized. The capsid proteins provide the anti-termination factor required for cRNA synthesis. The cRNA is then copied back into minus-strand viral RNA, which is incorporated into virions. Orthomyxoviruses, which possess a segmented minus-strand RNA genome, likewise synthesize two classes of plus-strand RNA from the same minus-strand RNA template. However, the mRNAs and cRNAs of these viruses are different at both their 5′ and 3′ ends (Fig. 15). As indicated earlier, the orthomyxoviral mRNAs possess nonviral sequences from capped host mRNAs at their 5′ ends and terminate 18–22 nucleotides prior to the 3′ end with the addition of poly(A) sequences. However, the orthomyxoviral template cRNAs are complete copies of vRNA from end to end without any nonviral sequence at the 5′ end or poly(A) sequences at the 3′ end. Therefore, for orthomyxoviral cRNA synthesis to occur, the viral polymerase must be able to initiate RNA synthesis without any host capped primer at the 5′ end and the RNA synthesis must not terminate until the complete 3′ end is reached, thus fully copying the entire template vRNA from the 3′ end to the 5′ end and without any polyadenylation. Such complete cRNAs then function as templates for vRNA (minus-strand) synthesis.

b. ***Replication of Double-Stranded Viral RNA***. Each segment of double-stranded viral RNA genome is replicated independently. First, the genome is transcribed to generate plus-strand mRNAs within the incoming virion core by the virion-associated RDRP. Next, the mRNA is used as a template by RDRP to

synthesize the minus RNA strand, and thereby mRNAs become converted into double-stranded RNA, which is then packaged into progeny virion capsids.

c. ***Replication of RNA via a DNA Intermediate.*** Retroviruses contain a diploid genome consisting of two identical RNA molecules, a tRNA primer (Fig. 1), and a reverse transcriptase, an RNA-dependent DNA polymerase, which also possesses both RNase H and integrase activity. Conversion of the plus-strand viral RNA into double-stranded DNA is initiated by viral reverse transcriptase using the tRNA as a primer. The RNA-dependent DNA replication process is complex and requires strand switching twice (Fig. 18). Eventually, a double-stranded proviral DNA is made in the cytoplasm, which is translocated into the nucleus and becomes integrated into the host genome. The integrated proviral DNA is then transcribed by the host RNA *pol* II into full-length plus-strand RNA, and that full-length RNA is then transported into the cytoplasm and encapsidated into progeny virions.

VI. ASSEMBLY AND MORPHOGENESIS OF VIRUS PARTICLES

As indicated earlier, when compared to either eukaryotes or prokaryotes, viruses use a unique multiplication strategy to produce their progeny. All cells, either prokaryotic or eukaryotic, multiply as a whole unit from parent to progeny and in a geometric order, that is, from 1 to 2 to 4 to 8, and so duplicatively on. Viruses, on the other hand, do not multiply as units. Rather, they are assembled from component parts. Each component part of progeny virus particles is made separately, and they are often made in different amounts and at different locations and compartments within the host cell. These viral components are then put together to form the whole (infectious) virus particles (virions). In this assembly-line type of process, all individual viral components need not be assembled at the same time, and in fact, some components may be put together separately to form higher-ordered structures, that is, subviral particles (e.g., capsid), before they are assembled into a whole progeny virus particle. The number of steps involved and the complexity of the assembly process may vary greatly from one type virus to another. Some viruses, like the polioviruses, have only a few components to assemble, and yet others, like the pox or herpesviruses, have many components to assemble and their assembly compared to polioviruses is a far more complex process involving multiple steps.

With respect to the assembly processes, viruses can be classified into two major subclasses: naked viruses and enveloped viruses. Naked viruses consist of a nucleocapsid only (i.e., the capsid containing the genome and no envelope). The assembly of the protein capsid and incorporation of genomic nucleic acid into the capsid to create this nucleocapsid will render the virus particle infectious. For

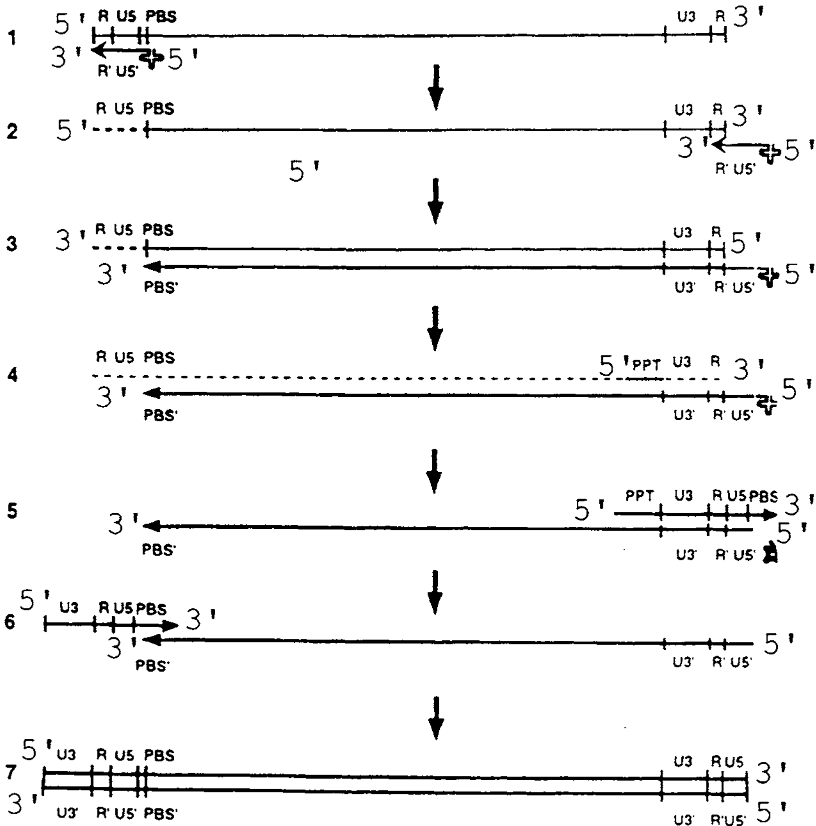


Fig. 18. Reverse transcription of retroviral genomic RNA into double-stranded proviral DNA. **Step 1**: Annealing of primer tRNA (shown as a cross-shaped symbol) to the primer-binding site (PBS), and synthesis of minus-strand strong-stop DNA. The R and U5 RNA is degraded by the RNase H activity of the reverse transcriptase, and the strong-stop DNA is released. **Step 2**: The first strand switch (or transfer). The minus-strand strong-stop DNA is annealed to the 3′ terminus of the genomic RNA via R–R′ hybridization (first strand jump). **Steps 3,4**: Further synthesis of minus-strand DNA, during which the genomic RNA is further degraded by RNase H. However, a small piece of RNA (the polypurine tract [PPT]) remains undergraded and serves as a primer for synthesis of plus-strand strong-stop DNA (step 5). **Step 5**: Termination of plus-strand DNA synthesis at 18 nucleotides into the primer tRNA, thus generating the new primer-binding site (PBS) sequence; the plus-strand DNA is then released from the minus-strand DNA. **Step 6**: The second jump (or the second transfer). The plus-strand strong-stop DNA is annealed to the 3′ terminus of the minus-strand DNA via PBS–PBS hybridization, completing the second jump. **Step 7**: Completion of the synthesis of the double-stranded proviral DNA. R = terminally redundant identical sequences at the 5′ and 3′ ends of viral RNA; U5 = unique nucleotide sequences near the 5′ end of the viral genome between the R and PBS (primer-binding site); U3 = the region near the 3′ end of the viral RNA between the initiation site of the plus-strand DNA synthesis and R sequences; PPT = polypurine tract that escapes RNase H digestion and serves as a primer for the second-strand DNA synthesis. Strong-stop DNA is the DNA copy of the region between the primer-binding site (PBS) and the 5′ end of the viral RNA genome. Reprinted with permission from Mak and Kleiman (1997).

these viruses, the virus receptor-binding proteins are part of the capsid proteins. Enveloped viruses, however, are those in which the nucleocapsid is surrounded by a lipid membrane containing the transmembrane viral proteins. In enveloped viruses, one of the transmembrane viral proteins (and not the capsid protein) contains the receptor-binding protein.

A. Assembly of Naked Viruses

The assembly of naked viruses occurs either in the cytoplasm (most RNA viruses) or nucleus (DNA viruses). For cytoplasmic viruses (e.g., the plus-sense RNA picornaviruses), the entire genomic RNA is translated into a single giant polyprotein (Fig. 2) that is cleaved by a virus-specific protease into P1 (a coat precursor protein); P1 is then cleaved by the protease 3C into VP0, VP3, and VP1 (5-S promoter). Five subunits (5-S promoter) — each containing one molecule of VP0, VP3, and VP1 — then assemble into pentamers (14 S). Twelve pentamers form the 60-subunit protein shell (capsid) for the picornaviruses. The viral RNA genome is then incorporated into the capsid, forming what is called the "provirion." Subsequently, the molecules of VP0 in the provirion are cleaved into molecules of VP4 and VP2 (Fig. 2), converting the provirion nucleocapsid into an infectious virion. This process of picornaviral capsid assembly is basically a self-assembly process whose rate is dependent on viral protein concentration.

For assembly of a naked virion to occur inside the nucleus, one of at least two distinct strategies can be used. The first of these would require that all capsid proteins, after their translation in the cytoplasm, must be transported into the nucleus either independently or cooperatively by forming a complex with other capsid proteins and that nucleocapsid assembly occurs around the viral genome in the host nucleus. This option is used by papovaviruses, whose DNA genomes, or minichromosomes, contain a single closed circular duplex DNA molecule complexed with cellular histone, which is organized into a nucleosome within the host nucleus. Papovaviral capsid assembly then proceeds in a stepwise fashion around the viral minichromosome. The capsid of SV40, which is a member of this virus group, contains 360 copies of its major viral protein (VP1) assembled into 72 pentamers plus 30 to 60 copies of internal proteins VP2 and VP3. VP2 contains the full VP3 sequence plus 100 extra amino acids at the NH_2 terminus, which are critical for interacting with the SV40 minichromosome. The papovaviral capsid proteins and minichromosomes assemble first into 200-S structures called provirions, which then mature into infectious virions. During this maturation, H1 histone protein is removed from the viral minichromosome and degraded.

Adenoviruses use a second type of strategy in which the capsid shell is first formed by the assembly of viral capsid proteins. Viral DNA, including core

proteins, is then inserted into the empty capsid shells to form infectious virions. Both of these nuclear DNA viruses as well as the cytoplasmic naked RNA viruses are primarily released to the extracellular environment by cell lysis.

B. Assembly of Enveloped Viruses

The assembly of enveloped viruses is much more complex than that of naked viruses. It involves not only nucleocapsid formation but also envelopment of the nucleocapsid and budding of virions from different cellular organelles and membranes. Subsequently, the virus is released into the extracellular environment. The assembly and the budding site on the cellular membrane varies with different groups of viruses. Some viruses, like the poxviruses and rotaviruses, bud from the endoplasmic reticulum (ER), while others, like the bunyaviruses, bud from the Golgi complex, and still others bud from the nuclear membrane, like the herpesviruses. Still other viruses (e.g., orthomyxo-, paramyxo-, rhabdo-, and retroviruses) use the plasma membrane (apical or basolateral) as the budding site.

Since the assembly processes of orthomyxo- and paramyxoviruses have been well studied, the steps involved in morphogenesis of these viruses will be discussed below in some detail. For comparison, rhabdovirus and retrovirus assembly will also be included as needed. First, I will discuss the steps that are common to both orthomyxoviruses and paramyxoviruses and then later point out the differences between these two viruses in terms of assembly and morphogenesis. As noted earlier, orthomyxo- and paramyxoviruses are enveloped RNA viruses that contain single-stranded RNA genomes of negative (minus) polarity, and they are assembled into nucleocapsids that have helical symmetry (Table I). Electron-microscopic studies have demonstrated that these viruses bud from the plasma membrane into the outside environment and that complete virions are usually not observed inside the cell during the productive infectious cycle.

1. Steps Common for Enveloped Virus Morphogenesis

For elucidating viral assembly and budding processes, the viral structure can be separated into three major subviral components, each of which must be brought to the assembly site for morphogenesis. These subviral components are: (a) the viral nucleocapsid (or viral ribonucleoprotein [vRNP]) containing the vRNA, NP (nucleoprotein), and transcriptase complex, which together form the inner core of virus particle; (b) the matrix protein, which forms an outer protein shell around the nucleocapsid and constitutes the bridge between the envelope and nucleocapsid; and (c) the envelope (or membrane), which forms the outermost barrier of these enveloped virus particles, containing the virally coded transmembrane pro-

teins and host cell lipids. Each of these virus groups (namely, orthomyxo-, paramyxo-, rhabdo-, and retroviruses) buds from the plasma membrane of infected cells. However, while the orthomyxo- and paramyxoviruses bud from the apical plasma membrane of polarized epithelial cells, both *in vivo* (e.g., in bronchial epithelium) and in cultured polarized epithelial cells (e.g., MDCK cells), the rhabdoviruses and retroviruses bud from the basolateral surface.

With respect to the processes involved in virus assembly, there are two major differences between orthomyxo- and paramyxoviruses: (a) Since the viral genome of orthomyxoviruses is segmented, multiple RNA segments (8 separate RNA segments for influenza types A and B, 7 RNA segments for influenza type C viruses) must be incorporated into infectious virions, whereas only one large RNA molecule is packaged in infectious paramyxovirus particles. (b) Since the transcription and replication of orthomyxoviral RNA and assembly of these viral nucleocapsids (vRNP) occur in the host nucleus, the viral nucleocapsids must be exported out of the nucleus into the cytoplasm for the final stages of viral assembly and for budding. In contrast, for paramyxoviruses, all of these steps, including assembly of viral nucleocapsids, take place in the cytoplasm.

Analysis of the steps involved in putting these subviral components together in an orderly fashion into an infectious virus particle is critical to an understanding of the assembly and budding processes. Two steps are obligatory for virus assembly and morphogenesis to occur. First, all of these viral components (or subviral particles) must be directed and brought to the assembly site, that is, the apical plasma membrane in polarized epithelial cells for assembly and budding of orthomyxo- and paramyxoviruses. Obviously, this step is the first obligatory requirement in virus assembly, since, if different viral components are misdirected to different locations or parts of the cell, virus assembly and morphogenesis cannot take place. Second, the viral components must interact with each other to form the proper virus structure during morphogenesis. It is possible that viral components may be directed to the assembly site but that defective interaction among these components will not yield infectious particles. However, although these two steps are obligatory, they alone may not be sufficient to form and release infectious virus particles. Therefore, virus components may be directed correctly to the assembly site and then interact with each other to form virus particles, yet infectious viruses may not be released into the medium. Such abortive virus morphogenesis in HeLa cells infected with influenza viruses has been observed where virus particles are formed on the plasma membrane but not released (Gujuluva *et al.*, 1994). Furthermore, each of these steps is indeed a complex multistep process.

Among the viral components, the greatest amount of information is available about the transport, sorting, and targeting of viral transmembrane proteins to the assembly site (prospective budding site) in the plasma membrane. It has been observed that the transmembrane viral proteins in virus-infected cells also preferentially accumulate at the virus assembly site, that is, the orthomyxo- and paramyxoviral proteins like HA, NA, F, and HN accumulate on the apical plasma

membrane, whereas rhabdoviral and retroviral transmembrane proteins like the VSV G and the HIV gp160 proteins are targeted to the basolateral plasma membrane in virus-infected polarized epithelial cells (see Blau and Compans, 1996; Tucker and Compans, 1993). Furthermore, it was also observed that, when these transmembrane viral proteins were expressed alone from cloned cDNAs in the absence of any other viral protein, they also exhibited the same phenotype as in the virus-infected cells, that is, depending on the viral protein, they also accumulated on either the apical or basolateral plasma membrane in polarized epithelial cells. These results demonstrated that these transmembrane viral proteins do not need the presence of any other viral proteins for their successful targeting to the assembly site, and that they possess the necessary determinants in their protein structure for their sorting and targeting to the correct cell surface (apical or basolateral) in polarized epithelial cells. Furthermore, these studies indicated that the sorting and localization of transmembrane proteins may be a critical factor in selection of the assembly site of virus particles and also may be a critical determinant in viral pathogenesis. For example, the wild-type Sendai virus (a mouse pathogen), which buds from the cell's apical surface, is pneumotropic and less virulent as compared to a pantropic and highly virulent mutant Sendai virus that buds from both apical and basolateral surfaces (see §VI.C).

Although the nature of the cellular machineries that target either viral or cellular proteins to their appropriate apical or basolateral plasma membranes are not fully understood, it is now accepted that these viral proteins are targeted directly to apical or basolateral surfaces and that for apical versus basolateral targeting protein sorting occurs during the process of vesicular transport from the trans-Golgi network (TGN) to the plasma membrane. It has also been shown that separate sorting machineries (vesicles) are involved in targeting to the apical and basolateral surfaces. It is therefore expected that viral proteins must possess determinants that selectively interact with either the apical or basolateral sorting machineries of the host cells. Those proteins that are directed to the basolateral membrane, including the VSV (a member of family Rhabdoviridae) G protein, possess basolateral sorting determinants in their cytoplasmic tail region. However, the nature and location of the sorting determinant(s) of apical proteins are more complex. For example, type I apical viral proteins like influenza virus hemagglutinin (HA) and type II transmembrane proteins, like influenza virus neuraminidase (NA), have been shown to possess at least two apical sorting signals that function independent of each other (see Lin *et al.*, 1998; Kundu *et al.*, 1996). One of these apical determinants is in the ectodomain of the protein (the part of the membrane protein that is exposed to the outside). Glycans are the likely candidates for the apical sorting determinants present on protein ectodomains. In addition, another apical determinant is present in their transmembrane domain (the part of the protein that spans the distance between the inner and outer layers of the lipid membrane and anchors the protein to the lipid bilayer via lipid–protein interactions). The mechanisms by which these proteins are targeted to the apical plasma membrane are unclear, although the transmembrane domains of apical type I and

II proteins may interact with Triton X-100 (TX100) detergent-insoluble lipid rafts, enriched in cholesterol and glycosphingolipids (GSLs). These rafts may function as platforms for targeting lipids and proteins to the apical plasma membrane (Scheiffele *et al.*, 1997).

In addition to the transmembrane glycoproteins, other viral proteins and subviral complexes such as matrix protein and nucleocapsid must also reach the assembly site. How these viral components are also directed to the assembly site is not yet fully understood. The possibility exists that the matrix (M1) protein may be directed to the assembly site by its interaction with the transmembrane viral proteins using a piggyback mechanism. Similarly, viral nucleocapsids (or ribonucleoprotein) may be transported to the assembly site either independently or on the back of the M1 protein. A number of studies have shown that matrix proteins as well as matrix protein/nucleocapsid complexes interact with the glycoproteins in transit to the plasma membrane (see Nayak, 1996). Furthermore, it appears that cytoskeletal components are involved in these processes, by interacting with both the matrix proteins as well as the nucleocapsids (including the nucleoprotein [NP]) and thereby facilitating transport of these components to the assembly site (see Avalos *et al.*, 1997).

The viral matrix protein is a key component in virus assembly and morphogenesis. It is the most abundant protein in virus particles and is the rate-limiting component in particle formation since the virus particle is greatly reduced when matrix protein synthesis is defective or reduced. However, particles with reduced amounts of glycoproteins can be formed efficiently, although such viruses may be less infectious, or noninfectious. Freeze-fracture electron microscopy has shown that, during bud formation in virus-infected cells and in virus particles, the matrix protein is present as a sheet between the lipid bilayer and the viral nucleocapsid. The results of these and other studies imply that the matrix protein is likely to interact with both the lipid bilayer of the membrane and its associated viral glycoproteins on the outer side, and with the viral nucleocapsid on the inner side of the virus particles. In virus particles, the matrix protein remains bound strongly to the nucleocapsid under conditions where membrane glycoproteins can be dissociated using nonionic detergents. It is only after further treatment with either low-pH (influenza virus) or high-salt (paramyxovirus) buffer that matrix proteins can dissociate from the viral nucleocapsids. Therefore, we have strong evidence of interactions between the nucleocapsids and the matrix proteins. However, a detailed analysis of the exact nature of these associations in virus-infected cells at different stages of the assembly process has yet to be undertaken.

Likewise, the location of the matrix protein and glycoproteins in virus particles and in virus-infected cells predicts an interaction between the matrix protein and glycoproteins. However, the interaction of matrix proteins with membrane glycoproteins has been rather difficult to demonstrate. Studies using morphological as well as biochemical analyses have demonstrated that the Sendai virus (a member of family Paramyxoviridae) M (matrix) protein can bind independently to either Sendai virus membrane glycoproteins (F or HN), and that this interaction can take

place on the plasma membrane as well as during exocytic transport of F and HN proteins through the Golgi complex. Biochemical and morphological studies have also shown that interaction of the Sendai virus M protein with viral glycoproteins can occur in the absence of nucleocapsid (or NP) protein (Sanderson *et al.*, 1994). However, the nucleocapsid protein may facilitate further with membrane association of the M protein by interacting with viral glycoproteins. In Sendai virus-infected cells, the nucleocapsid possibly becomes membrane associated with the help of the M protein acting as a bridge between the nucleocapsid protein and the membrane glycoproteins. The fact that virus particles can be formed in the absence of one or more of their membrane glycoproteins suggests that interaction of the matrix proteins with all transmembrane viral proteins is not obligatory for viral morphogenesis to occur. Furthermore, influenza virus particles can be formed, albeit inefficiently, which contain HA and NA proteins that lack the cytoplasmic (inner membrane side) of the tail (Jin *et al.*, 1997). However, these tail-minus influenza virus particles exhibited extremely high pleomorphism and bizarre morphological shape, suggesting an impairment of interaction between matrix proteins and glycoproteins. Similarly, in VSV-infected cells, virus particles are formed that possess VSV G protein with a foreign cytoplasmic tail, supporting the hypothesis that the interaction of matrix protein with the cytoplasmic glycoprotein tail is not an obligatory requirement for morphogenesis (Schnell *et al.*, 1998).

Although the majority of host proteins present on the cell membranes are excluded during the budding process, two classes of host components (the lipids and cytoskeletal components) do appear to be involved in virus morphogenesis. Viral lipids are directly borrowed from the cellular lipids, and depend on the site of virus budding. For example, viruses budding from the apical or basolateral membrane will incorporate the lipids present on one specific side of the plasma membrane. It has also been specifically suggested that glycosphingolipids (GSLs) and cholesterol, which are present in the host cell apical membrane, may play a specific role in targeting viral transmembrane proteins to the apical membrane domains (budding sites). For example, both type I (influenza virus HA, Sendai virus F) and type II (influenza virus NA, Sendai virus HN) proteins associate with TX100 detergent-insoluble lipid rafts enriched in GSL and cholesterol during exocytic transport to the viral assembly sites on the plasma membrane.

Cytoskeletal components facilitate both the transport of viral proteins to the assembly site and the budding process. Subviral components such as the NP proteins of nucleocapsids, as well as the RNA of the influenza viral RNP and the matrix proteins, interact with the host cytoskeletal components during intracellular transport. Cytoskeletal components, particularly microtubules and microfilaments, are known to be involved in targeting proteins to apical and basolateral membrane domains. Actin filaments have been observed in budding paramyxoviruses, and are also present in released viral particles of both orthomyxo- and paramyxoviruses. These observations suggest that the host cytoskeletal elements are actively involved in the assembly and budding processes of virus particles. However, studies that attempted to examine these roles by using cytoskeletal disruptive agents like

cytochalasins B and D have produced conflicting results. Some of these studies have reported drug-induced enhancement of virus assembly and release, while others found a decrease in or no effect on virus replication following treatment with these agents. It is likely that both the nature of host cells and viruses as well as the timing of the addition of these drugs in the infectious cycle may have contributed to the variable results obtained in these studies. For example, drugs added relatively early in the infectious cycle might interfere with transport of viral components and thereby inhibit virus assembly and release, whereas, if these drugs were added late in the infectious cycle, they might enhance virus release by disrupting microfilaments and facilitating closure of viral buds.

2. *Assembly of Paramyxoviruses*

For paramyxoviruses, three viral components — namely, the NP of viral nucleocapsids (RNP), the M protein, and two transmembrane glycoproteins (F and HN) — are critically important for the assembly and morphogenesis of virus particles. However, the function of some other viral proteins such as P or L in the assembly process is unknown. These latter two proteins are synthesized independently from different viral messengers, and in different cellular compartments than NP, M, F and HN, and their synthesis appears to be temporally regulated. NP, the major component of the viral capsid, is critically required for both genome replication and nucleocapsid formation. Both free (soluble) and nucleocapsid-bound NP are present in the cytoplasm of virus-infected cells. Viral genome (vRNA) synthesis is coordinated with NP synthesis such that vRNA synthesis will not take place in the absence of a sufficient level of free NP proteins. Both the M and NP proteins are synthesized on free polyribosomes in the cytoplasm. NP associates with vRNA to form the RNP complex, and M proteins interact with the vRNP to form the M/vRNP complex. These proteins and complexes are formed in the cytoplasm. These M/NP (or M/RNP) complexes as well as M and NP proteins become associated independently with cytoskeletal components.

These components then interact with the F and HN glycoproteins either individually or together during their transport through the exocytic pathway or following insertion of F and HN into the plasma membrane, or both. The site of interaction between the M/RNP complex and the viral glycoproteins is essentially regulated by the presence and availability of these viral membrane glycoproteins in specific membrane components. For example, late in the infectious cycle in virus-infected cells, the majority of the viral glycoproteins are already present on the plasma membrane; therefore, the M/RNP–glycoprotein interaction is likely to occur predominantly on the plasma membrane. In addition, Sendai virus M protein can interact with Sendai virus glycoproteins in the absence of NP protein or RNP during exocytic transport of glycoproteins (Sanderson *et al.*, 1994). Although some M proteins can interact alone but inefficiently with lipid membranes, viral glycoproteins greatly facilitate membrane association of M protein, suggesting a direct interaction of M proteins with membrane-inserted viral glycoproteins. On the plasma membrane, M proteins will also interact with each other, forming a sheet of M proteins. This sheet of M proteins then acts to exclude, from viral budding sites,

other cellular membrane proteins, except for viral glycoproteins, which are retained since they are bound to the M protein. This M protein sheet binds to the virus nucleocapsid and becomes the assembly site for budding. Nucleocapsids are directed to the assembly site by cytoskeletal components such as actin, which may also aid in bud formation. Eventually, the bud containing the vRNP (nucleocapsid) and matrix protein will be closed by fusion of membrane lipids. Experimental evidence supports a presumed interaction of M proteins with themselves and with viral RNP and glycoproteins, as well as interaction of M and NP (or RNP) with the cellular cytoskeleton elements (Sanderson *et al.*, 1995). However, the mechanism of bud formation and the forces causing membrane curvature for initiating bud formation remain unclear. It has been proposed that the interaction among the M proteins and pushed by actin filaments may be responsible for bud formation. In some cases, actin filaments are seen to extend into the viral bud. Therefore, closure of the bud seemingly would require disassembly of actin and might result in incorporation of some actin into the virus particles. Experimental evidence for the presence of actin within virus particles does support this proposal for the role of actin in bud formation and virus release. However, nothing is known about the specifics of actin disassembly during the process of budding and release of virus particles from the cell.

3. *Assembly of Orthomyxoviruses*

The assembly of orthomyxoviruses (influenza viruses, Fig. 12) differs from that of paramyxoviruses in two unique ways. First, unlike paramyxoviruses, influenza vRNP is synthesized in the nucleus. Therefore, vRNPs must exit from the nucleus into the cytoplasm. Second, eight separate vRNP segments must assemble into one infectious virus particle, requiring that multiple vRNP segments must either uniquely or randomly associate with each other in such a way that during virus assembly at least one of each of the eight separate vRNP segments is incorporated into every infectious budding progeny virus.

After uncoating, the infecting vRNPs that have entered the cell are transported into the nucleus for replication and transcription of viral RNAs, following which the progeny vRNPs are formed in the nucleus. In order for assembly of progeny influenza viruses to occur, the newly formed vRNPs must be transported out of the nucleus into the cytoplasm and then directed to the assembly site on the plasma membrane. However, since the NP protein possesses nuclear localization and nuclear retention signals, it is not clear what enables the vRNP to exit the nucleus. Although it appears that dissociation of the incoming M1 matrix protein in the acid pH of endosomes during the viral uncoating process is necessary in order for the vRNPs to subsequently enter nucleus, massive association of M1 with progeny vRNP in the nucleus is not required for exit of vRNPs from the nucleus into the cytoplasm. A study performed in ts51 (temperature-sensitive M1 mutant WSN of influenza virus) virus-infected cells has revealed that progeny vRNPs exit from the nucleus into cytoplasm at the restrictive temperature when the ts51 M1 protein is not functional, and the majority of this mutant's newly synthesized M1 proteins is retained in the nucleus. The ts defect is due to hyperphosphorylation of the M1

protein at the restrictive temperature. Recently, NS2 protein has been shown to provide the nuclear export signal for exit of viral RNP from the nucleus into the cytoplasm (O'Neill *et al.*, 1998). However, unlike such other nuclear export proteins as REV protein of HIV, NS2 does not have an RNA binding domain.

The following scenario is envisioned for the nuclear exit of vRNP (Nayak, 1996). In the nucleus there are likely two classes of vRNPs, of which one class is involved in active transcription and replication of viral RNA. This class of progeny vRNPs are not exported from the nucleus. A second class of progeny vRNPs that are to be exported from the nucleus are rendered transcriptionally inactive and sequestered from the transcriptionally active vRNPs. The support for incorporation of the transcriptionally inactive progeny vRNPs in virus particles comes from an observation that in released progeny virus particles there are polymerase molecules present only at one end of the vRNP that are not present all over of the vRNP, as would be expected if those progeny vRNPs had been undergoing active transcription within the nucleus during their exit from the nucleus and if such vRNPs were assembled into the progeny virions. Furthermore, the requirement of a 5′ primer for initiation of transcription and incorporation of that primer into the transcription product mRNA, as observed during *in vitro* studies, would also support the concept of initiation of transcription rather than chain elongation. On the other hand, simple chain elongation without a primer requirement would be expected to occur if instead the vRNPs had essentially been "frozen" during active transcription when they exited from the nucleus and budded into virus particles from the infected host cell. These results provide further evidence to support the hypothesis that only transcriptionally inactive vRNPs are exported from the nucleus. However, it is not clear how the progeny vRNPs are rendered transcriptionally inactive in the nucleus. Since M1 is known to inhibit transcription, it is possible that a few M1 molecules bind at the critical site on either the progeny vRNA or the viral polymerase, and that these M1 molecules then render those vRNPs transcriptionally inactive and sequester them from the transcriptionally active vRNP that are retained in the nucleus. NS2 then may bind to the transcriptionally inactive vRNPs and facilitate their export to the cytoplasm. Such a hypothesis can explain how in ts51-infected cells vRNPs are exported from the nucleus even though the majority of M1 is retained in the nucleus. Only a few M1 molecules will be able to block transcription of vRNPs and bind to NS2 for nuclear export of vRNPs.

Once vRNPs are exported from the nucleus into the cytoplasm, the subsequent sequence of events leading to assembly and budding have yet to be resolved for orthomyxoviruses. Late in the infectious cycle, the majority of the cytoplasmic M1 proteins in virus-infected cells become membrane bound immediately after their synthesis. Pulse-chase experiments have shown that late in the infectious cycle the newly synthesized M1 protein incorporated quickly into the progeny virus particles, suggesting that M1 is a rate-limiting factor in virus morphogenesis and release. This result also suggests that viral RNPs and glycoproteins are already present on the plasma membrane of virus-infected cells late in the replication cycle

at the time when M1, soon after its synthesis, interacts with the membrane-bound nucleocapsid complexes. However, the interaction of M1 with membrane-associated components is not totally dependent on having viral glycoproteins associated with the membrane. This independence has been demonstrated by the fact that free M1 alone can become membrane bound and, possibly, even diffuse to the assembly site, where it becomes stably associated with the membrane after it interacts with other previously bound molecules of M1, membrane glycoproteins, and vRNPs. However, despite this evidence, the precise role of the viral components (namely, M1, glycoproteins, and nucleocapsids), as well as the sequence of steps involved in the association of M1 with the membrane during influenza virus assembly and budding, have yet to be determined. Since progeny virus particles can be formed that lack either the HA or NA proteins or lack the cytoplasmic tail of the HA or NA, it would seem that the association of M1 with any one of these glycoproteins is sufficient to allow for viral morphogenesis and that M1 may interact with the transmembrane domain as well as cytoplasmic tail of either of these glycoproteins. On the other hand, since only a few molecules of M2 protein are found in the virus particle, M2 is unlikely to be a major factor in membrane association of M1 or in assembly and morphogenesis of virus particles.

Finally, how the eight different RNA (or RNP) segments are incorporated into each infectious virus particle remains unclear. Two models have been proposed: (a) selective assembly of eight unique vRNP segments, and (b) random assembly of multiple vRNP segments into a virus particle. The latter model would propose that more than eight RNA segments are incorporated randomly into each particle, so that a fraction of virus particles will possess eight separate RNA segments and be infectious. The majority of data from genetic and biochemical experiments does not differentiate between these two models. Selective assembly predicts that each virus particle will possess only eight separate RNA segments. However, extra vRNA segments have been shown to be present in virus particles under forced selection, suggesting that eight vRNA segments can be randomly incorporated into virus particles. This, however, may represent only a minority of virus particles. On the other hand, an extrapolation of findings that describe the loss of a homologous vRNA segment in defective interfering (DI) virus particles would suggest that multiple segments of the same RNA segment are not favored for incorporation into the same virus particle and would therefore favor some selective process for achieving incorporation of specific vRNP segments into a virion during viral assembly. However, there are as yet neither definitive data nor a specific hypothesis to explain the mechanism by which selective incorporation of vRNPs in a virus particle is accomplished. Finally, the pleomorphism and plasticity of virus particles (Fig. 6) as well as a high particle-to-infectivity ratio (20 to 1000 particles/pfu) can accommodate either a selective or random mode for the strategy of vRNP assembly during viral morphogenesis (see Lamb and Choppin, 1983).

Last, after budding from the host cell, viruses must be released into the surrounding medium and spread outward to infect other cells. Some viral components are critically involved in the viral release process. The data from ts viruses at

restrictive temperature clearly demonstrate that the viral neuraminidase (NA) protein is involved in virus release. The NA removes the sialic acid, which is the receptor for influenza virus, from membrane glycolipids and glycoproteins of both the virus and the virus-infected cells, and thus prevents self-aggregation among virus particles and reattachment to the virus-infected cell.

C. Role of Viral Budding in Viral Pathogenesis

The site and nature of budding can be an important contributory factor in viral pathogenesis, particularly for such respiratory viruses as the influenza and Sendai viruses. The influenza and Sendai viruses bud from the apical surface of polarized epithelial cells (e.g., bronchial epithelial cells) into the lumen of the lungs and are therefore usually pneumotropic, that is, restricted to the lungs, and do not cause viremia or invade other internal organs. However, occasionally, some influenza viruses — like fowl plague (H5 or H7) viruses (H5 or H7 indicates hemagglutinin subtype specificity of type A influenza viruses) and WSN (H1N1) viruses — are not restricted to the lungs and produce viremia infecting other internal organs (pantropism) and cause a high degree of mortality in infected animals. In humans, most of the influenza viruses are pneumotropic and do not spread to other internal organs. However, it is not clear if the Spanish Influenza of 1918, the most devastating influenza pandemic in recorded human history, which killed 20 to 40 million people worldwide, particularly affecting young healthy adults, was only pneumotropic. In addition to pneumonia, some people died due to massive pulmonary hemorrhage and edema (see Taubenberger, 1998). The 1918 flu virus, like fowl plague, may have caused viremia and infected other organs. Therefore, it is possible that highly virulent viruses may not be restricted to lungs in either chicken or humans. H5N1, the Hong Kong chicken virus, is extremely virulent and pantropic for chicken, causing viremia and spreading to other internal organs. In 1997, H5N1 was shown to have infected at least 16 peoples, including 5 who died of complications from the influenza infection. The majority of them had clinical pneumonia (or acute respiratory distress syndrome), gastrointestinal symptoms, and impaired hepatic and renal function. Although the virus did not spread from human to human or spread very inefficiently, the high morbidity and mortality in humans raises the question as to the cause for such high virulence in the infected individuals. The viruses that are restricted to lungs are called "pneumotropic," whereas the viruses that cause viremia and spread to other internal organs are called "pantropic." Why some influenza viruses are pneumotropic and others are pantropic is an important question for predicting the outcome of a major influenza epidemic or pandemic.

The severity of viral pathogenesis depends on both viral and host factors, including host immunity. The virulence determinants of influenza viruses are complex and multigenic. However, one factor that has been thought to be critical in viral growth and virulence is the cleavability of HA $\rightarrow$ HA1 and HA2. Influenza virus is normally restricted to the lungs because its HA can be cleaved by tryptase Clara,

a serine protease restricted to the lungs. However, some HA that contains multiple basic amino acids at the HA1–HA2 junction, as is found only in H5 and H7 avian subtypes, can be cleaved by furin and subtilisin-type enzymes, which are present ubiquitously. Therefore, such viruses can grow in other organs. In addition, the NA of some influenza viruses (e.g., WSN viruses) binds and activates plasminogen into plasmin in the vicinity of HA, and the activated plasmin cleaves HA → HA1 and HA2, rendering the virus infectious. Therefore, WSN virus, which lacks multiple basic residues in its HA, can grow and multiply in tissues other than the lungs.

However, although the cleavage of HA → HA1 and HA2 is a major virulence factor, it is not the only factor contributing to the pantropism of a normally pneumotropic flu virus. For example, although WSN virus is pantropic and neurovirulent in the mouse, gene reassortment experiments demonstrated that the WSN NA gene responsible for the cleavage of HA was not sufficient for neurovirulence in chickens or mice. Other WSN genes, like the M and NS genes, in addition to the NA gene, were required for neurovirulence and, therefore, likely pantropism. The function of M and NS genes in neurovirulence is not known. The M gene in Sendai virus has been shown to affect apical versus basolateral budding and contribute to the pantropism of F1-R Sendai virus mutant (Tashiro and Seto, 1997). Therefore, it is possible that, in addition to increased cleavability of HA, the pantropic virus causes alteration in apical budding, releasing more virus basolaterally. Since blood vessels are proximal to the basolateral surface of cells, basolateral budding would facilitate more viruses entering into the blood, causing viremia, and invading other internal organs. Therefore pantropic influenza viruses like WSN/33 virus or highly virulent Hong Kong H5N1 and H7N1 fowl plague viruses may also cause altered budding from apical and basolateral surfaces. Thus, altered budding may be considered an important trait for the virulence of a specific strain of influenza virus. However, the role of altered budding in influenza virus pathogenesis remains to be determined.

Sendai virus, like influenza virus, is a pneumotropic mouse virus that buds apically. However, a Sendai virus mutant, F1-R, which exhibited pantropism possessed two characteristics (Tashiro and Seto, 1997): (1) cleavage mutation of F → F1 and F2 ubiquitously due to the presence of multiple basic residues, and (2) altered budding from both the apical and basolateral surfaces. On the other hand, the Sendai virus mutants that exhibited only cleavage of F → F1 and F2 or that exhibited only altered budding did not cause viremia or pantropism in the mouse. This would also support the argument that altered budding may be a factor that facilitates viremia and pantropism. Therefore, altered apical versus basolateral budding could be an important factor in dissemination of virus into the blood, invasion of internal organs, pantropism, and the higher virulence of a specific virus strain.

VII. CONCLUSIONS

The replication and morphogenesis processes of viruses are different from those of either prokaryotic or eukaryotic organisms. In this chapter, I have presented

some of the general steps involved in the viral infectious cycle, including: entry, uncoating, transcription, translation, replication, and assembly processes, and the possible role of budding in viral pathogenesis. Of these, viral morphogenesis is the most obscure phase in the virus life cycle. Yet, knowledge of how the particles are formed during this morphogenetic stage is fundamental to understanding virus growth and multiplication, and therefore is crucial in defining viral infectivity, transmission, virulence, tissue tropism, host specificity, and pathogenesis, and contributes to an overall understanding of the disease process and progression of disease, including host morbidity and mortality. In addition, the site of budding can affect virus virulence and pathogenesis. Elucidation of the viral replicative and assembly processes is critical in terms of enabling us to find ways to block these steps and thereby intervene in the viral life cycle and disease process. Much remains to be done to achieve these goals, particularly in terms of elucidating those stages of the viral assembly process that relate to how viral components are brought to the assembly site, how those components interact with each other at the assembly site, and how viral budding actually occurs. A better understanding of viral replication and morphogenesis may lead us to develop novel therapeutic agents capable of interfering with these critical steps in viral multiplication and pathogenesis.

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