VIRAL RNA POLYMERASES

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I. INTRODUCTION

The presence of a DNA-dependent RNA polymerase in virions of poxviruses was first demonstrated in 1967 by Kates and McAuslan[1] and Munyon et al.[2] Among the various DNA virus groups, the cytoplasmic DNA viruses, i.e., viruses which grow in the cytoplasm of infected cells, contain a DNA-dependent RNA polymerase in virions, but viral RNA polymerase has never been found for other DNA viruses, which replicate in the cell nucleus, including adenoviruses, herpesviruses, papovaviruses, and parvoviruses. These viruses contain infectious DNA that can be transcribed by host cell DNA-dependent RNA polymerases.

In 1970, Baltimore et al.[3] demonstrated that virions of vesicular stomatitis virus (VSV) contain an RNA-dependent RNA polymerase activity. In the early 1970s, the virions of a number of animal RNA virus groups were found to contain virus-specific RNA polymerases, including arenaviruses, bunyaviruses, orthomyxoviruses, paramyxoviruses, reoviruses, and rhabdoviruses. The genomes of these viruses are either double-strand RNA or single-strand RNA with opposite polarities to their mRNAs, i.e., minus-strand or negative-strand RNA. In addition, the virions of retroviruses were found to contain an RNA-dependent DNA polymerase known as reverse transcriptase.[4,5] These findings led to a dramatic change in the conception of viruses, that is, viruses are not simple capsules of genetic information but they contain some apparatuses involved in enzymatic reactions characteristic of all cellular organisms.

The original classification of the modes of virus genome expression was presented in 1971 by Baltimore,[6] who grouped the animal viruses into six groups on the basis of differences in the genome structure and the mode of transcription into mRNA. As a definition, mRNA was assigned + polarity for all classes. The genome classes transcribed into mRNA are Group 1, double-strand DNA (+/− DNA); group 2, single-strand + DNA, from which mRNA is transcribed through +/− DNA intermediates; group 3, double-strand RNA (+/− RNA); group 4, single-strand + RNA; group 5, single-strand − RNA; and group 6, single-strand + RNA, which is reproduced through double-strand +/− DNA intermediates.

In terms of replication strategy, the single-strand RNA viruses can be divided into two groups, plus-strand viruses (group 4) and minus-strand viruses (group 5). In the case of the plus-strand (or positive-strand) viruses, infection is initiated with the translation of the parental genomic RNA that leads to the production of the viral RNA polymerase. This enzyme synthesizes minus-strand templates, plus-strand genomes and, in some cases, plus-strand subgenomic messages for virion structural proteins. By contrast, the minus-strand (or negative-strand) viruses introduce a virion-associated RNA polymerase into the host as a component of nucleocapsid cores, and the initial event in virus replication is primary transcription by the parental nucleocapsids to produce messages for all virus-encoded products.

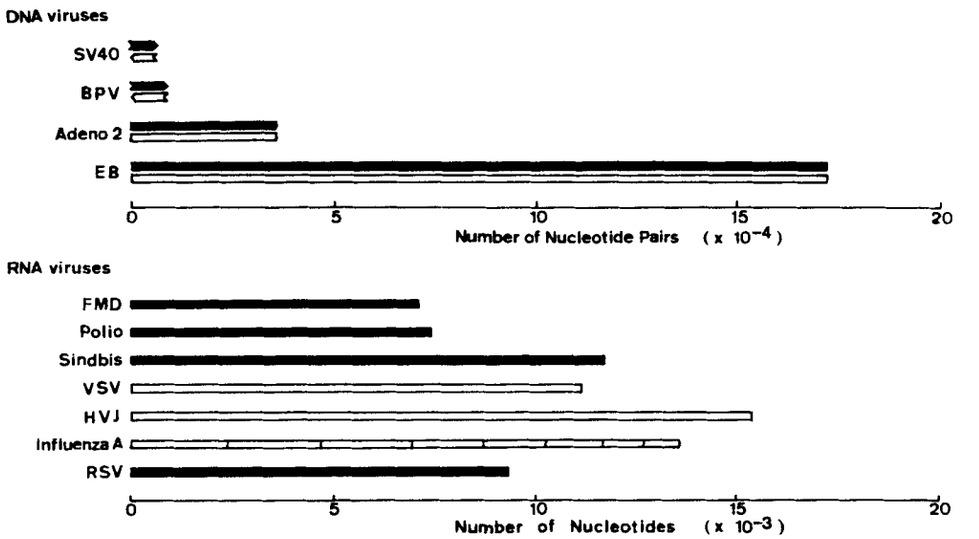

FIGURE 1. The sizes of viral genomes. The sequences of viral genomes have been determined for a number of animal DNA and RNA viruses. The genome sizes of some type of viruses are illustrated. SV40, simian vacuolating virus 40; BPV, bovine papilloma virus; Adeno 2, human adenovirus type 2; EB, Epstein-Barr virus; FMD, foot-and-mouth disease virus; Polio, poliovirus type 1; VSV, vesicular stomatitis virus; HJV, hemagglutination virus of Japan (or Sendai virus); Influenza A, influenza virus A/PR/8/34; RSV, Rous sarcoma virus. SV40 and BPV, both belonging the Papovaviridae, carry covalently closed supercoiled DNAs, which are shown as linear forms. The filled bars represent positive strands and the open bars represent negative strands.

Subsequent events include the synthesis of complementary plus-strand template and of genomic minus-strand RNA and amplified or secondary transcription.

The early studies related to the finding and preliminary characterization of virus-associated RNA polymerase were reviewed by Raghow and Kingsbury[7] in 1976 and Bishop[8] in 1977. In recent years, our knowledge of virus-specific RNA polymerases has increased markedly. The exciting breakthroughs appeared in particular with the development of rapid techniques for the cloning of viral genes and for determining the nucleotide sequences of the cloned genes. Such information has given an enormous impact on our understanding of not only the molecular nature of self-replicating DNA and RNA molecules but also the apparatuses involved in these processes. This review summarizes the up-to-date knowledge of the structures and functions of viral RNA polymerases and shows that the viral RNA polymerases are complex in both structure and function, being composed of multiple subunits and have multiple functions, the functions being controlled through structural changes. Due to space limitations, we have focused primarily on animal viruses and have presented little on RNA-dependent DNA polymerases (or reverse transcriptases) associated with retroviruses. We have attempted to provide references which allow easy access to both the primary literatures and earlier reviews related to viral RNA polymerases. Finally, fundamental questions are also provided, into which we are just beginning to get some insights. We hope that this review will stimulate the study of viral RNA polymerases.

A. Cloning and Sequencing of Viral Genomes

The most important recent progress in the field of basic virology was the elucidation of the DNA and RNA sequences of the genomes for a number of animal viruses. The complete sequences have been determined for prototype viruses of most major families, including the Adenoviridae, Herpesviridae, Hepadnaviridae, Papovaviridae, and Parvoviridae of DNA virus groups, and the Flaviviridae, Orthomyxoviridae, Paramyxoviridae, Picornaviridae, Retroviridae, Rhabdoviridae, and Togaviridae of RNA virus groups (Figure 1). After the

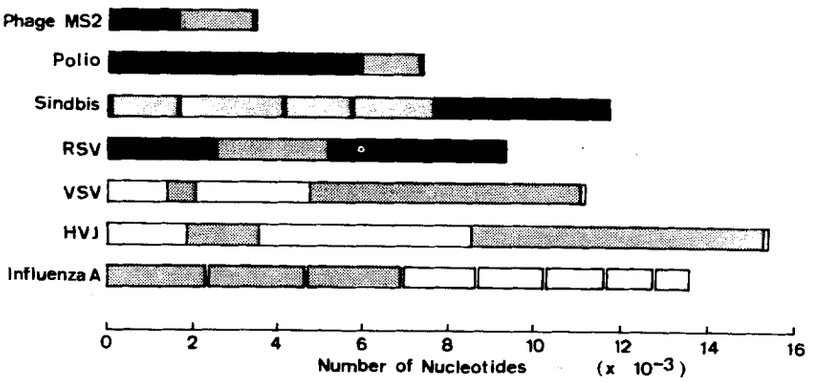

FIGURE 2. Coding assignments of viral genomes and the genes for RNA polymerase proteins. The genes encoding viral RNA polymerase (or DNA polymerase in the case of RSV) have been estimated for various RNA viruses and are illustrated by the meshed bars on the respective genetic maps. The filled bars represent plus strands, while the open bars represent minus strands. MS2, RNA phage MS2; Polio, poliovirus type-1; Sindbis, sindbis virus; RSV, Rous sarcoma virus; VSV, vesicular stomatitis virus; HVJ, the hemagglutination virus of Japan (or Sendai virus); Influenza A, influenza virus A/PR/8/34.

pioneering DNA sequence determinations in the cases of SV40,[9] polyoma viruses,[10,11] and human papovavirus BK[12,13] of Papovaviridae, the DNA sequences have been determined for papilloma viruses,[14-16] parvoviruses,[17,18] hepatitis B viruses,[19-22] Woodchuck hepatitis viruses,[23] adenoviruses,[24] and adeno-associated virus.[25] Recently, the DNA sequences of 172,282 and 124,884 bp (base pairs) have been determined for Epstein-Barr virus[26] and varicella-zoaster virus (VZV),[27] respectively, of the Herpesviridae.

On the other hand, the complete sequences of the RNA genomes have been determined for prototypes of most animal RNA virus families. Among the Picornaviridae, the RNA sequences have been determined for several strains of poliovirus,[28-34] rhinovirus,[35-36] and foot-and-mouth disease virus.[37-39] The complete sequences of 11,703 and 10,862 nucleotides were determined for the positive-strand RNA genomes of sindbis virus of the Togaviridae,[40] and yellow fever virus[41] and West Nile virus[42-44] of the Flaviviridae, respectively. Among negative-strand RNA viruses, the complete sequences have been determined for 11,162 nucleotides of VSV of the Rhabdoviridae,[45-47] 15,383 nucleotides of HVJ (hemagglutination virus of Japan or Sendai virus) of the Paramyxoviridae,[48,49] and 13,588 nucleotides of the sum of all eight segments from influenza virus A/PR8.[50-56] The sequence of either viral RNA or proviral DNA is known for more than 20 virus species of retroviruses.

The sequence determination confirmed that the sizes of RNA genomes are strikingly smaller than those of DNA genomes (Figure 1). The largest contiguous RNA genomes belong to coronaviruses with sizes of about 20,000 bases, which are one tenth the sizes of the largest viral DNA genomes. RNA molecules exceeding this length may lose their identity due to the low fidelity of the replication apparatuses for RNA genomes (e.g., see Reference 57).

B. Sequences of Viral RNA Polymerase Genes

The sequence determinations have provided a better and broader understanding of the structure, function, and biosynthesis of viral proteins. In light of this information, it is likely that a major part of the viral sequence is devoted to make enzymes involved in duplication of the viral genomes (Figure 2). For example, the RNA polymerase genes make up 19.5, 62.7, 54.5, and 49.3% of the poliovirus, VSV, HVJ, and influenza virus genomes, respectively.

Comparison of the genome sequences revealed that homologous sequences exist between

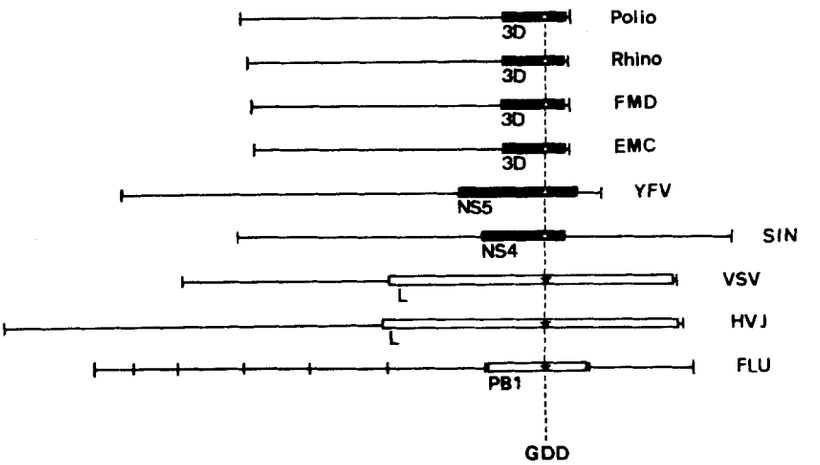

FIGURE 3. The RNA polymerase genes in viral RNA genomes. In the genomes of most, if not all, RNA viruses there are genes which contain canonical RNA polymerase sequences centered with a tripeptide, Gly-Asp-Asp (GDD). The filled bars represent positive strands, while the open bars represent negative strands.

DNA-dependent RNA polymerases from eukaryotic cells and DNA viruses (for details see Section II); the homology also exists among viral RNA-dependent RNA polymerases, including the canonical Gly-Asp-Asp (GDD) sequence[58,59] (Figure 3). The homology is not uniform along the viral genes but rather consists of several conserved domains interspersed with nonhomologous regions. Since the same tripeptide sequence exists in the genes for RNA-dependent RNA polymerases of plant and bacterial RNA viruses, it is believed that the RNA viruses have diverged from a common ancestor. Similar tripeptide sequences exist even in the genes for RNA-dependent DNA polymerases or the reverse transcriptases of retroviruses. This domain might therefore be involved in the recognition of template RNAs.

C. Purification and Characterization of Viral RNA Polymerases

Following the initial burst of work demonstrating the association of viral RNA and DNA polymerases for the members of eight major virus groups (reviewed in References 7,8), i.e., arenaviruses, bunyaviruses, orthomyxoviruses, paramyxoviruses, poxviruses, reoviruses, retroviruses, and rhabdoviruses, attention was switched to the purification and characterization of the polymerases.

The molecular mechanism of RNA synthesis by viral RNA polymerases was studied in in vitro systems using virions, isolated nucleocapsis cores, and solubilized RNA polymerases. So far, several species of viral RNA and DNA polymerases have been purified from virions and characterized in detail, including a DNA-dependent RNA polymerase from vaccinia virus of the Poxviridae,[60-62] an RNA-dependent RNA polymerase from VSV of the Rhabdoviridae,[63,64] and RNA-dependent DNA polymerases from a number of retroviruses.[65,66] Solubilization and partial purification has also been performed for influenza viruses of the Orthomyxoviridae[67,68] and Newcastle disease virus (NDV) of the Paramyxoviridae.[69] These studies suggest that virus-specific RNA polymerases are complex in both structure and function; they consist of multiple subunits and carry multiple functions, as summarized in this review. Furthermore, these studies revealed that viral factors, which suppress the expression of RNA polymerase activity, are present in virions of some viruses.[70,71] It is also clear that some viral RNA polymerases require certain cellular cofactors before they can function.[72-74] Such a host cofactor requirement could result in the specificity of a virus for one form of differentiated cell over another.

II. DNA-DEPENDENT RNA POLYMERASES OF DNA VIRUSES

The Poxviridae is a large family containing the largest of all animal DNA viruses that infect both vertebrates and invertebrates (for a review see Reference 75). On the basis of their replication and transcription strategy, poxviruses are classified as "group 1" viruses.[6] Poxviruses contain long double-strand linear DNA genomes with variable-length inverted terminal repetitions. Recently, it was found that the two strands are covalently linked at both termini.[76,77] The vaccinia virus, a prototype of the Poxviridae, is one of the best-characterized viruses in this family. Its main components are 90% protein, 5% lipid, and 3% DNA. Its genome is a double-strand DNA, with a chain length of about 187 kbp (kilobasepairs), which directs the synthesis of more than 100 viral polypeptides. Its virions are composed of more than 100 polypeptides, which can be detected by two-dimensional gel electrophoresis.

A. RNA Polymerase of Vaccinia Virus

The gene expression of animal DNA viruses depends on the cellular DNA-dependent RNA polymerases in the infected cells. Poxviruses are, however, exceptional because they contain their own transcriptional apparatuses (for reviews see References 78 and 79). Therefore, poxviruses can grow in the cytoplasm of infected cells and even in enucleated cells.[80,81] A number of viral specific enzymes have been identified, including a DNA-dependent RNA polymerase, mRNA-modifying enzymes, a DNA-dependent DNA polymerase, and more than ten enzymes related to nucleic acid metabolism.[78,79] Most of these enzymes are packaged in the viral particles, and their enzymatic activities can be expressed using virions after solubilization of viral envelopes with lipid solvents. Vaccinia virus has been considered an ideal model system for analyses of the molecular mechanisms of transcription and replication in eukaryotes. This review focuses on the structures and functions of the viral RNA polymerase and the enzymes involved in the modification of primary transcripts into functional molecules.

1. Purification, Structure, and Function

The original idea that vaccinia virus contains its own transcriptional apparatus was based on the fact that it is able to grow in enucleated cells.[80,81] The presence of RNA polymerase activity in virions was indeed demonstrated on incubation of viral cores in the presence of ribonucleoside triphosphates.[1,2] The vaccinia viral RNA polymerase was, however, purified first from virus-infected cells.[60] Cellular RNA polymerases were recovered in the nuclear fraction, but the viral polymerase was found in the cytoplasm of virus-infected HeLa cells. Starting from a cytoplasmic extract, the viral RNA polymerase was purified by chromatography on DEAE-Sephadex® and phosphocellulose columns, followed by glycerol density gradient centrifugation, with a 3.1% yield. The vaccinia enzyme behaved differently from cellular RNA polymerases on chromatography and centrifugation. On SDS-polyacrylamide gel electrophoresis (PAGE), the viral enzyme was dissociated into equimolar amounts of seven putative subunits, of 135, 130, 77, 34, 19.5, 16.5, and 13.5 kdaltons, and several minor bands (Table 1). The sum of the molecular weights of the major subunits was 425 kdaltons, which agreed with the molecular weight of the native enzyme, as determined by glycerol centrifugation. The three largest subunits seemed to correspond to the viral structural polypeptides, VP1c, VP1d, and VP2c, suggesting that the viral RNA polymerase in infected cells is similar, if not identical, to the virus core-associated RNA polymerase.

Later, two groups succeeded in the solubilization and purification of the viral RNA polymerase from virions. Baroudy and Moss[61] extracted the enzyme by treatment of virions with 0.2% sodium deoxycholate, 10 m*M* dithiothreitol, and 0.1 *M* or a higher concentration of NaCl, and purified it by chromatography on DEAE-cellulose, DEAE-agarose, phospho-

Table 1
SUBUNIT STRUCTURE OF VACCINIA VIRAL RNA POLYMERASE

Source	Subunit ($M_r \times 10^{-3}$)	Ref.
Infected cells	135, 130, 77, 34, 19.5, 16.5, 13.5	60
Virions	140, 137, 37, 35, 31, 22, 17	61
Virions	137, 137, 34, 31, 20.5, 18.5, 17	62

cellulose, and aminopentyl-agarose columns to near homogeneity, as judged on PAGE under nondenaturing conditions. The molecular weight of the native enzyme was approximately 500 kdaltons, as determined on glycerol gradient centrifugation. The enzyme was composed of seven subunits of 140, 137, 37, 35, 31, 22, and 17 kdaltons (Table 1). The combined molecular weight of 517 kdaltons was in agreement with that of the native enzyme. This subunit composition was similar to that of the enzyme isolated from infected cells except that the 77 kdaltons polypeptide was present only in the enzyme from infected cells. It is not known yet whether this component is an essential subunit with a regulatory function or a contaminant.

Spencer et al.[62] also reported the purification of a viral RNA polymerase from vaccinia virus. The procedure they used for the enzyme solubilization was similar to that used by Baroudy and Moss.[61] The enzyme was purified over 23,000-fold by chromatography on DEAE-cellulose and phosphocellulose columns, with a 64% yield. The purified enzyme was composed of seven subunits of 137 (doublet), 34, 31, 20.5, 18.5, and 17 kdaltons (Table 1). The subunit composition was similar to those already reported.

When cell lines containing α-amanitin-resistant RNA polymerase II were infected with vaccinia virus, virus production took place normally even in the presence of the drug.[82,83] Irradiation with γ-ray at doses capable of inhibiting the host cell transcription did not affect the multiplication of vaccinia virus.[82-84] Removal of the cell nucleus, UV inactivation of the nucleus, or addition of α-amanitin, however, reduced the production of mature virions of vaccinia virus.[84] These observations suggested that the host cell RNA polymerase II is not essential for but somehow affects the growth of vaccinia virus. The immediate and rapid inhibition of cellular RNA synthesis after virus infection was not due to decreased uptake of substrates or precursors for RNA synthesis, reduced synthesis of nucleoside triphosphates or enhancement of RNA degradation, but was due to progressive and eventual loss of the cellular RNA polymerase activity.[85] The results of in vitro experiments demonstrated that the ability to transcribe the adenovirus VA gene, the gene transcribed by the host DNA-dependent RNA polymerase III, was still retained by extracts of vaccinia virus-infected cells; in contrast, the extracts of infected cells could not support the transcription of the genes for the RNA polymerase II, for example, the adenovirus major late gene and SV40 genes.[86,87] To account for all of these observations, it was hypothesized that the host cell RNA polymerase II was disassembled after vaccinia virus infection, and then one or more of the polymerase subunits were assembled together with the virus-coded polymerase subunits into a functional form of viral RNA polymerase.

In agreement with this hypothesis, Morrison and Moyer[88] reported recently that a subunit of the host cell RNA polymerase II was directly associated with an RNA polymerase of rabbit pox virus. They prepared three different monoclonal antibodies: the first one reacted only with the virus-coded 137-kdalton polypeptide,[89] the second one reacted with the 170-kdalton subunit of RNA polymerase II from rabbit kidney cells (and the 220-kdalton subunit of HeLa cell RNA polymerase II), and the third one cross-reacted with both the viral and cellular antigens. Both the 170-kdalton cellular and 137-kdalton viral polypeptides also

immunologically cross-reacted with the antibody against the 220-kdalton subunit of *Drosophila* RNA polymerase II. Both purified virions and the purified viral RNA polymerase contained a polypeptide that cross-reacted with a second-type antibody. The results of immunofluorescence studies revealed the translocation of this polypeptide from the cell nucleus to the virosome, the site of viral RNA synthesis in infected cells. Finally, the viral RNA polymerase activity was immunoprecipitated by treatment with the second-type antibody. These observations indicated that the largest subunits of viral and cellular RNA polymerases are identical or there is some structure homology between the two proteins. Recently, the gene encoding the largest subunit of the vaccinia virus RNA polymerase was cloned and sequenced.[90] From the amino acid sequence, a molecular weight of 146,967 was predicted for this polypeptide. When the amino acid sequence was compared with those of the largest subunits of yeast RNA polymerases III and II[91] and *Escherichia coli* RNA polymerase,[92] homology was found in five separate domains; in addition, a sixth homologous domain was present in the vaccinia and yeast RNA polymerases which was absent in the *E. coli* RNA polymerase. The most highly conserved sequence among these four RNA polymerases was the seven amino acid sequence, Asn-Ala-Asp-Phe-Asp-Gly-Asp, starting from position 413 of the vaccinia viral enzyme. The largest subunit of *Drosophila* RNA polymerase II also contains this sequence.[93] The function of the largest subunit of the viral enzyme might be analogous to that of the largest subunits of cellular RNA polymerases. The β′ subunit of *E. coli* RNA polymerase[94] and the largest subunit of RNA polymerase II from Ehrlich ascites tumor cells[95] are DNA-binding components. The vaccinia enzyme lacked the carboxy terminal heptapeptide repeat sequence of Pro-Thr-Ser-Pro-Ser-Tyr-Ser, which is common in eukaryotic RNA polymerase and believed to play a role in the recognition of DNA signals common to eukaryotes.[91] Supporting this concept was the finding that the largest subunit (180 kdaltons) of RNA polymerase IIB was a truncated version of the 220-kdalton subunit of RNA polymerase IIA lacking this repeat;[91] accurate transcription from promoters was catalyzed only by the IIA (II0) form enzyme.[96]

2. Template Specificity and Promoter Selectivity

The purified RNA polymerase from vaccinia virus-infected cells could transcribe single-strand DNA and poly[d(AT)] more efficienty than double-strand DNA;[60-62] however, the crude enzyme transcribed double-strand DNA as well.[60] Supercoiled DNA was also an active template, yet its template activity was abolished by relaxation of the DNA by DNA topoisomerase.[62] A good correlation was found between the RNA-synthesizing and DNA-binding activities, the order being single-strand DNA > supercoiled DNA > double-strand DNA. The high rate of transcription of the supercoiled DNA template might be due to the presence of locally denatured regions in such DNAs. The crude enzyme appeared to contain a factor(s) which allowed the RNA polymerase to transcribe double-strand DNA. A factor(s) that induces a single-strand portion in a specific region of double-strand DNA with a higher-ordered structure could be such a transcription regulatory factor.

The purified enzyme exhibited no preference for DNA promoters; the whole length of vaccinia viral DNA was equally transcribed. In contrast, only one half of the viral genome was transcribed in vitro by the viral core-associated enzyme[97] or in vivo during the early phase of virus infection. The in vitro transcription system using permealized virions exhibited selective transcription of early genes.[1,2,98]

Golini and Kates[99] developed faithful in vitro transcription system using template-free soluble extracts of virions. This system allowed the correct initiation of transcription from viral early promoters on supercoiled as well as linear viral DNA, producing capped and polyadenylated transcripts. The results of enzyme fractionation and reconstitution experiments demonstrated that a virion-associated factor other than the viral RNA polymerase was involved in the specific transcription initiation,[100] although it remained to be elucidated whether the factor was virus coded or host derived.

Among the variety of templates examined so far, both poly(dA) and poly(dG) are inactive templates,[61] suggesting that the RNA polymerase is unable to initiate transcription with pyrimidine nucleotides as is the case for all known RNA polymerases. The high Km values for GTP (333 μM) and ATP (80 μM) compared with that for CTP (12 μM) supported this interpretation. In fact, both mRNA synthesized in vitro by vaccinia virus cores and mRNA isolated from virus-infected cells were found to carry either A or G residues in 5′ cap structures.[101] High concentrations of ATP are required for RNA synthesis by crude enzymes or viral cores, but not by the purified viral RNA polymerase, suggesting that ATP is utilized in a yet-unidentified process(es) besides as a substrate for RNA polymerization.[102,103] AMPPNP and ATPγS could be utilized as substrates for RNA synthesis, but the substitution of these ATP analogues for ATP prevented the accurate transcription in a soluble extract system. Both unwinding of duplex DNA and migration of the transcription complex on duplex DNA might be ATP dependent. Concerning this, it is noteworthy that vaccinia virus exhibits a number of ATP-dependent enzyme activities, including those of DNA- or RNA-dependent phosphohydrolases.[102,103,105,106]

An in vitro transcription system for specific genes was then developed by Puckett and Moss,[86] in which whole cell extracts prepared from vaccinia virus-infected cells supported accurate and specific transcription from a cloned DNA fragment carrying the 7.5 K (K represents 1000) protein gene, one of the early genes. The results of nuclease S1 analysis indicated that the start site of in vitro RNA synthesis was identical in every respect with that of in vivo transcription. The use of a series of deletion mutant DNAs as templates revealed that a DNA sequence between 230 bp upstream and 30 bp downstream of the transcription start site was needed for this transcription. Mapping and sequencing of the 7.5 K gene revealed that there was an AT-rich 60-bp DNA sequence immediately upstream of the transcription initiation site.[107-110] AT-rich sequences have always been found in promoters such as the Pribnow box (TATAAT) in prokaryotes and the Hogness-Goldberg box (TATAAA) in eukaryotes. In fact, a 275-bp fragment containing both the transcription initiation site and this AT-rich sequence supported efficient expression of a variety of genes, including the genes for herpesvirus thymidine kinase,[111] hepatitis B virus surface antigen,[112] influenza virus hemagglutinin,[113] and chloramphenicol acetyltransferase,[114] when they were inserted in the proper orientation upstream of the respective gene bodies. It was therefore concluded that the 275-bp fragment contained a promoter recognized by the vaccinia viral RNA polymerase. Several promoters of vaccinia virus genes have since been mapped and sequenced, such as the promoters for the genes encoding three early proteins, a 19-kdalton polypeptide (19 K gene),[115] a 42-kdalton polypeptide (42 K gene),[115] and thymidine kinase (TK gene),[116] and for a major late gene encoding a 28-kdalton polypeptide.[117] The early genes contained an AATAA sequence at about the -15 position upstream of the respective initiation sites and a TATA sequence at about the -40 position, the two signals being separated by 20 to 24 bp.[118] Weir and Moss[117] proposed two signals, a nonanucleotide sequence including AATAA and an octanucleotide sequence including TATA, as the early promoters. In the promoter region of the late gene, a sequence of eight consecutive A residues was found at the -15 position, but a sequence similar to the consensus nonanucleotide sequence was not found at the -40 position.[117]

To characterize the in vitro function of various viral promoters, a recombinant virus system has been developed, which contained the chloramphenicol acetyltransferase (CAT) gene body.[114,117] When an early promoter is inserted, the CAT activity is detected immediately after infection with the recombinant virus. On the other hand, when a late promoter was inserted, the enzyme activity is detected only after the onset of DNA replication and this induction is prevented by the addition of cytosine arabinoside, an inhibitor of DNA replication.[119] A conventional plasmid vector system containing the CAT gene preceded by the vaccinia virus promoters was also employed. The CAT activity was expressed only on

transfection into vaccinia virus-infected cells.[120] These observations indicated that the promoter fragments contain cis-acting regulatory elements responding to the temporal control of gene expression during the virus growth cycle and, moreover, led to the concept that there is a trans-acting factor(s) that recognizes the DNA signal(s) and regulates transcription. Selective transcription of early genes was observed in a truncated DNA-directed in vitro transcription system prepared from purified virions.[100] Therefore, virions contain all the factors necessary for the transcription of the early genes but lack a factor(s) needed for transcription of the late genes. The putative trans-acting factor(s) specific for the late gene transcription might be induced in virus-infected cells.

B. RNA Modification Enzymes in Poxviruses

The infectious vaccinia virus contains a transcriptase system capable of synthesizing functional mRNA,[97] that is polyadenylated,[121] capped, and methylated,[101] indicating that virions contain enzymes in RNA modifications. The structures and functions of the RNA modification enzymes are summarized.

1. Poly(A) Polymerase

A poly(A) polymerase is present in poxviruses, which catalyzes the addition of adenylate residues derived from ATP to the 3′ ends of primers. Such a purified viral poly(A) polymerase has a native molecular weight of 80 kdaltons and is composed of 51- and 35-kdalton subunits.[122] From vaccinia virus-infected cells, two cellular and one virus-derived poly(A) polymerases have been purified.[123] One of the cellular enzymes, which is located in the nucleus and composed of a single polypeptide with a molecular weight of 75 kdaltons, is able to utilize both RNA and oligo(A) as primers; the second cellular enzyme is present in the cytoplasm and is able to add adenylates only to poly(A) chains, suggesting that the former enzyme is involved in the initiation of poly(A) addition and the latter in the elongation of poly(A) chains. The catalytic properties of the viral poly(A) polymerase are similar to those of the second cellular enzyme. The molecular weight of the native viral enzyme is 70 kdaltons, and it is composed of 57- and 37-kdalton subunits. The apparent difference in size between the virus-bound enzyme and the enzyme from infected cells might be due to modification(s) of the poly(A) polymerase after virus infection.

2. Capping Enzyme and Methyltransferases

The viral capping enzyme or GTP:mRNA guanylyltransferase catalyzes the formation of the blocked structure, GpppN, at RNA 5′ termini. The enzyme activity has been purified as a complex which also exhibits AdoMet:mRNA(guanine-7′-) methyltransferase activity. In this view, this complex with multiple functions is designated as the "capping enzyme complex". The molecular weight of the purified complex is 127 kdaltons, and it is composed of 95- and 31-kdalton polypeptides.[124,125] The sizes of the two subunits differed slightly among various preparations in different studies, i.e., 96 and 26 kdaltons,[126] 90 and 26 kdaltons,[127] and 95 and 28 kdaltons (plus 59 kdaltons).[128,129] The capping reaction consists of two consecutive transguanylation reactions: the formation of a covalently linked enzyme-GMP intermediate, followed by the transfer of the GMP moiety to the 5′ termini of mRNA. The GMP is linked to the 95-kdalton subunit via a phosphoamide bond.[130] In addition, an RNA 5′-phosphatase activity is associated with the capping enzyme complex, which removes the terminal γ-phosphate of nascent RNA molecules.[131] The resulting RNA with a diphosphate residue is the only guanylate acceptor used by the capping enzyme.

Since the 5′ termini of in vitro mRNA carry the cap-I structure, i.e., either m^7GpppGm or m^7GpppAm, virions also contain AdoMet:mRNA(ribose-2′-) methyltransferase, which catalyzes the methylation of ribose-2′ position. The purified enzyme has a molecular weight of 38 kdaltons; the acceptor for this reaction is almost exclusively m^7GpppA or m^7GpppG;

neither uncapped RNA nor capped RNA unmethylated at the guanine-7 position serves as an acceptor for this reaction.[132,133]

The sequence of the overall capping reactions can be summarized as follows:

$$pppNpNp(RNA) \rightarrow ppNpN(RNA) + Pi$$

$$pppG + E(capping\ enzyme\ complex) \rightarrow EpG + PPi$$

$$EpG + ppNpN(RNA) \rightarrow GpppNpN(RNA)$$

$$GpppNpN(RNA) + AdoMet \rightarrow m^7GpppNpN(RNA) + AdoHcy$$

$$m^7GpppNpNp(RNA) + AdoMet \rightarrow m^7GpppNmpN(RNA) + AdoHcy$$

The first four reactions are catalyzed by the capping enzyme complex, while the last reaction is catalyzed by the ribose-2′ methyltransferase. The commercially available capping enzyme is prepared from vaccinia viruses.

C. DNA Polymerase and DNA-Related Enzymes of Poxviruses

Among animal DNA viruses, those belonging to three families, poxviruses, herpesviruses, and adenoviruses, produce their own DNA-dependent DNA polymerases which are distinct from cellular DNA polymerases. These DNA polymerases are not structural components of virions but are induced in virus-infected cells. Replication of small DNA viruses, e.g., papovaviruses and parvoviruses, depends on cellular DNA polymerase α. The viral DNA polymerases have been purified from cells infected with vaccinia virus of the Poxviridae,[134] herpes simplex virus of the Herpesviridae,[135,136] and human adenovirus of the Adenoviridae.[137,197] The molecular weights of these DNA polymerases have been determined to be 110, 140, and 140 kdaltons, respectively. All these enzymes exhibit the intrinsic activity of 3′ to 5′ exonuclease, which is believed to be involved in proofreading. Physical mapping studies and genetic analyses of drug-resistant and temperature-sensitive DNA polymerase mutants allowed the location of the viral DNA polymerase genes for these viruses. The results of nucleotide sequence analyses indicated that the vaccinia virus, herpesvirus and adenovirus DNA polymerases were composed of 938,[138] 1235,[139,140] and 1056 amino acid residues,[141,142] respectively, the molecular weights of the respective enzymes being 108,577, 136,272, and 120,400. The deduced amino acid sequences of these viral DNA polymerases show a marked degree of homology.[138,143] A highly conserved 14 amino acid sequence with a six consecutive amino acid sequence, Tyr-Gly-Asp-Thr-Asp-Ser (YGDTDS), in the center exists starting at positions 720, 880, and 864 of the vaccinia virus, herpes simplex virus, and adenovirus DNA polymerases, respectively. The homology was found in the putative DNA polymerase gene of EB virus starting at position 749.[26] The 14 amino acid sequence might form a part of the functionally essential site for DNA synthesis. In fact, this region forms a reverse turn structure flanked by β-pleated sheet, which is often found in DNA-binding domains.[138,143] No significant homology was detected among bacterial DNA polymerases.

In addition to these virus-coded DNA polymerases, specific host factors are required for the replication of viral DNAs. The species of host factors required differ depending on the virus species. Nuclear factor I (NFI) is the most well-characterized host factor, which is essential for adenovirus DNA replication. NFI is a DNA-binding protein with affinity to sites containing a $TGGN_{6-7}GCCAA$ sequence.[144]

In addition to the DNA polymerase, a number of enzymes related to DNA or RNA metabolism are coded for by the vaccinia viral genome, most of which are assembled into virions. The DNA topoisomerase with a molecular weight of 44 kdaltons is a typical eukaryotic type I enzyme.[104] The DNA-binding protein with a molecular weight of 11 kdaltons

(VP11b) is one of the most abundant proteins in virions[145] and plays a role in the condensation of viral DNA during maturation of virions. Polyribonucleotide kinase,[146] phosphohydrolases (nucleoside triphosphatases),[102,105,106] deoxyribonucleases,[147,148] and protein kinase[149,150] are assembled in virions. Phosphohydrolase I with a molecular weight of 61 kdaltons catalyzes the single-strand DNA-dependent hydrolysis of either ATP or dATP. Its catalytic properties are similar to those of ATP-dependent DNA-unwinding proteins (reviewed in Reference 357), implying that it plays a role in the activation of duplex DNAs as templates for transcription. On the other hand, phosphohydrolase II catalyzes the single-strand DNA or RNA-dependent hydrolysis of all four ribonucleoside 5′-triphosphates and two deoxynucleotides, dATP and dTTP. Its catalytic properties are similar to those of bacterial transcription termination factor ρ. A protein kinase with a molecular weight of 62 kdaltons is associated with virions, which phosphorylates serine and threonine. The substrate protein for this protein kinase is not known yet.

III. RNA-DEPENDENT RNA POLYMERASES OF POSITIVE-STRAND RNA VIRUSES

Animal viruses containing single- and plus-strand RNAs as their genomes are classified as "group 4" viruses,[6] which comprise three nonenveloped virus families, i.e., picornaviruses, nodaviruses, and caliciviruses, and three enveloped virus families, i.e., togaviruses, flaviviruses, and coronaviruses. All these viruses encode the RNA-dependent RNA polymerases for transcription and replication and the RNA genomes. Retroviruses are also enveloped single- and plus-strand RNA viruses but encode the RNA-dependent DNA polymerases (or the reverse transcriptases) for the reverse transcription of viral RNAs into viral DNAs. The molecular mechanisms of transcription and replication have been studied extensively for poliovirus of the Picornaviridae.

A. RNA Polymerase of Poliovirus

Members of the picornavirus family are made up of nonenveloped virions approximately 22 to 30 nm in diameter with an icosahedral symmetry. Picornaviruses are divided into four genera: enteroviruses, cardioviruses, rhinoviruses, and aphtoviruses. All of these viruses are mammalian pathogens, and the enterovirus and rhinovirus groups contain a number of significant human pathogens (for reviews see References 151 and 152).

The type-1 poliovirus genome is a single-strand RNA molecule 7433 nucleotides long, which has a poly(A) tail of 80 to 120 nucleotides in length at the 3′ terminus and a covalently linked small protein, designated as VPg (viral genome-linked protein), at the 5′ terminus.[28,29,153,154] The minus-strand RNA contains a poly(U) tract at the 5′ terminus, indicating that the 3′ poly(A) is genetically encoded. The sequences have been determined for several different strains.[30-34] In the case of a type-1 poliovirus, the RNA contains a single long reading frame encoding a long polypeptide chain, designated as a "polyprotein", of 2207 amino acids (Figure 4). Translation starts at a single initiation codon at nucleotide 741 from the 5′ terminus and continues down to a termination codon at nucleotide 7361. The polyprotein is proteolytically processed at at least ten cleavage sites to yield individual products (reviewed in References 155 and 156). There are three functional regions, P1, P2, and P3 (the nomenclature is that adopted in 1983).[157] From the amino terminal (P1 or capsid protein) region, four capsid proteins, VP1, VP2, VP3, and VP4, are produced.[158] The protein shell of virions is made up of 60 copies each of these four virus-specific core proteins. The carboxy terminal (P3 or replicase) region contains the RNA-dependent RNA polymerase (protein 3D), VPg (protein 3B), and 3C protease. Product 2A is the second protease, which is encoded by the middle (P2) region. Three types of cleavage occur duing proteolysis of the polyprotein. The 3C protease cleaves the polyprotein at eight or nine Gln-Gly pairs.[159]

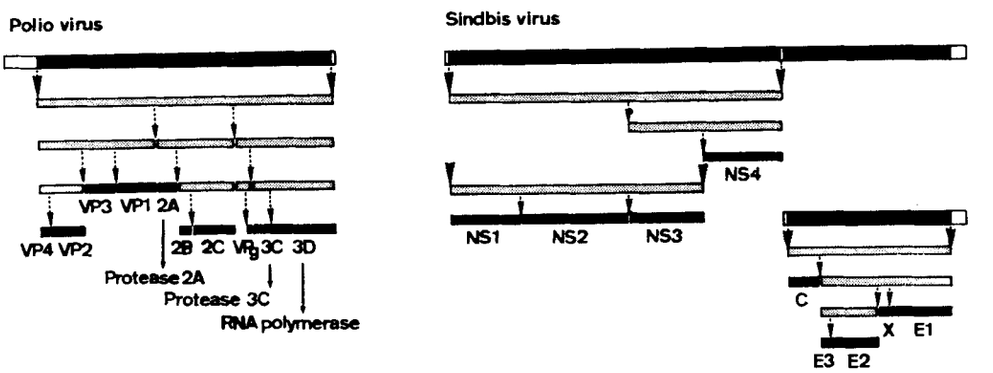

FIGURE 4. The cleavage maps of viral polyproteins. Polioviral RNA is translated into a single species of polyprotein, which is stepwisely processed to yield four structural proteins (VP1, VP2, VP3, and VP4) and several NS proteins, including the RNA polymerase and the polyprotein-processing proteases. Three proteases are involved in the polyprotein cleavage: two virus-coded proteases, 2A and 3C and one yet unidentified protease. Sindbis viral RNA is translated into polyproteins for four NS proteins (NS1, NS2, NS3, and NS4), whereas a polyprotein for structural proteins (C, E1, E2, and E3) is produced by translation of a subgenomic RNA, which is synthesized in virus-infected cells.

Cleavage of the P1-P2 and 3C-3D boundaries occurs at Tyr-Gly pairs due to the second protease 2A.[160] Cleavage of VP0 into VP4 (1A) and VP2 (1B) takes place between Asn and Ser, suggesting that a third viral protease or a cellular protease with a different specificity is involved in this process. In virus-infected cells, capsid proteins are produced more than other nonstructural proteins. The difference in the production levels of individual proteins could result from premature termination of translation or selective degradation. The organization and expression of RNA genomes are essentially identical for other picornaviruses.[35-39]

After poliovirus infection of susceptible cells, viral RNA is uncoated in the cytoplasm and, after removal of VPg from the 5′ terminus, translated into a single polyprotein, which is processed during translocation to both structural and nonstructural proteins, including the RNA-dependent RNA polymerase. The newly synthesized RNA polymerase transcribes the infected plus-strand RNA into the complementary RNA with minus-polarity, which is required for the amplification of the plus-strand RNA. Thus, the plus-strand RNA is used not only as mRNA for the production of viral proteins but also as the template for the production of progeny virions. The plus-strand RNA consists of more than 90% of viral RNA in infected cells.

1. Purification, Structure, and Function

RNA-dependent RNA polymerase is induced in the cytoplasm of poliovirus-infected cells.[162] The viral RNA polymerase was first identified in the cytoplasm of infected cells as an enzyme complex, which contains both the RNA polymerase and the endogenous viral RNA template. These RNA polymerase-viral RNA complexes are associated with the cytoplasmic membrane. Both the "replicative form" (RF) (template-transcript duplex) and the "replicative intermediate" (RI) (template associated with multiple strands of nascent transcripts) are recovered in this membrane complex. Moreover, RNA replication in vitro, i.e. the synthesis of plus-strand RNA, takes place only when the membrane fraction is used. Attempts have therefore been made to solubilize and purify the RNA-dependent RNA polymerase from the enzyme complex. When a crude lysate of poliovirus-infected cells was treated with LiCl, the RNA polymerase-viral RNA complexes sedimented with a sedimentation coefficient of 25S.[163,164] The enzyme activity was measured after adding a primer such as oligo(U).

The enzyme activity peak fraction contained a single major polypeptide with a molecular weight of 58 kdaltons, as determined by SDS-PAGE, which comigrated with viral protein 3D (Figure 4), suggesting that it is a virus-coded component of the viral RNA polymerase.[163] Flanegan and Baltimore[165] demonstrated that a poly(A)-dependent poly(U) polymerase activity could be solubilized from the membrane fraction of infected cells by treatment with a detergent and 2 *M* LiCl. The poly(U) polymerase activity was detected only when oligo(U) was added as a primer, suggesting that a factor donating a primer was present in virus-infected cells. On glycerol gradient centrifugation, the poly(U) polymerase sedimented with an apparent sedimentation coefficient of 4S, indicating that the RNA polymerase was no longer complexed with viral RNA. The 4S polymerase was comprised of a monomer of a 62.5-kdalton polypeptide designated as p63, which was considered to be the viral protein 3D (see Figure 4).

An RNA polymerase was also isolated from the soluble fraction of a cytoplasmic extract.[164] This enzyme, with a sedimentation coefficient of 7S, contained p63 and, in addition, a 77-kdalton polypeptide, which was considered to be a processing intermediate, 3CD (see Figure 4). The crude 7S polymerase was able to transcribe poliovirus RNA without an exogenously added primer.[166,167] Therefore, it appeared that the 7S polymerase contained not only the primer-dependent RNA polymerase but also a factor(s) capable of substituting for an oligo(U) primer. This was later designated as "host factor" and the enzyme fraction carrying these two activities was designated as an "RNA replicase".

Later, two groups succeeded in the isolation of an RNA polymerase devoid of the host factor.[72,168] The purified RNA polymerase consisted of only p63 (protein 3D) and was free from its precursor 3CD and other viral proteins.[169] When a primer or the host factor was added to the purified RNA polymerase, it regained the ability to transcribe viral RNA. Thus, it was concluded that viral protein 3D is the essential and presumably catalytic component of polioviral RNA polymerase.

In the presence of oligo(U) primers, the primer-dependent RNA polymerase could transcribe both poly(A) and polyadenylated RNA.[164-170] The product RNAs were covalently linked to the primers and associated with template RNAs, forming template-product duplexes.[167,168,170-173] In contrast, the RNA replicase, i.e., a mixture of the RNA polymerase and the host factor, could transcribe viral RNA in the absence of added primers.[72,174-177] The product RNAs, were, however, covalently linked to the template RNA, forming a snap-back structure, and therefore were twice the size of the template RNA.[167,172,173,178]

2. *Roles of Host Factors*

Dasgupta et al.[72] found that the addition of a salt-wash fraction of ribosomes from uninfected cells converted the primer-dependent RNA polymerase into a primer-independent RNA replicase. This activity was designated a "host factor". Since oligo(U) could substitute for the host factor, it was considered that the host factor played a role in the initiation of minus-strand RNA synthesis. In agreement with this hypothesis, antibodies against the host factor inhibited the template-dependent RNA replicase reaction but not the RNA chain elongation due to RNA polymerase-viral RNA complexes.[179]

Andrews and Baltimore[180] demonstrated that their host factor preparation catalyzed the uridylylation of short RNA fragments contaminating viral RNA preparations and the resulting uridylated fragments acted as primers for the synthesis of minus-strand RNA. The uridylated fragments were immunoprecipitated by anti-VPg antibodies, indicating that they were associated with viral RNA. On the other hand, Young et al.[172,178] reported that the largest product formed in the presence of the host factor was twice the size of the viral RNA template because the product RNA was covalently linked to the template RNA. These observations altogether supported a model that the host factor catalyzes the uridylylation of the viral RNA itself at its 3′ poly(A) tail; the poly(U) tail thus formed associates with the poly(A) sequence

through a snap-back mechanism and serves as a primer for the synthesis of minus-strand RNA.[176,180] As expected from this model, the host factor required the poly(A) tail for its action.

A partially purified eIF-2 was found to substitute for the host factor.[177] However, this fraction contained, besides eIF-2, two major proteins of 60 and 95 kdaltons in molecular weight, and a terminal uridylyltransferase (TUTase) activity. On glycerol gradient centrifugation, the host factor activity sedimented along with the TUTase activity but not with eIF-2. Highly purified eIF-2 did not exhibit the host factor activity, whereas the purified TUTase from HeLa cells was indistinguishable from the host factor by structural and functional criteria. The purified TUTase required UTP, Mg^{2+}, a sulfhydryl reagent, and an RNA primer. When poly(A) was added as a primer, four or five UMP residues were polymerized to the 3′ terminus, resulting in the formation of a hair-pin structure. It was therefore concluded that the TUTase is the host factor for polioviral RNA synthesis. As noted below, both minus- and plus-strand RNAs in infected cells are attached with VPg, a genome-linked viral protein, at their 5′ termini. Therefore, it remains unknown how VPg is attached to the 5′ end of the minus-strand RNA. One idea is that the dimeric RNA molecules are cleaved at the poly(A)-oligo(U) junction, and this reaction takes place together with the coupling with a covalent linkage of VPg to the cleaved RNA terminus. It cannot, however, be ruled out that such palindromic dimers are formed through a side-reaction and that VPg-primed initiation leads to the synthesis of minus-strand RNA.

The RNA replicases of RNA bacteriophages contain cellular EF-Tu and EF-Ts, both of which are the elongation factors for protein synthesis, as essential subunits. However, the host factor of the polioviral RNA replicase could not be replaced by any of the known eukaryotic translation factors.[72] The molecular weight of a partially purified host factor from an RNA replicase fraction was found to be approximately 72 kdaltons.[72] The host factor could also be purified from either a postmitochondrial supernatant[174] or a ribosomal salt-wash from uninfected HeLa cells.[175] The host factor from uninfected cells could bind to RNA polymerase coupled to Sepharose column.[174] A single polypeptide with a molecular weight of 67 kdaltons and an isoelectric point of 6.3 was found to be associated with the host factor activity.

The host factor prepared by Morrow et al.[181] exhibited two activities: a stimulatory activity toward viral RNA synthesis due to the primer-dependent RNA polymerase and a protein kinase activity. The protein kinase phosphorylated a number of proteins, including the α-subunit of eukaryotic protein synthesis initiation factor 2(eIF-2) and the host factor itself. Both activities were inhibited by antibodies against the host factor. Kinetic studies demonstrated that the phosphorylation of the host factor preceded viral RNA synthesis; reincubation of the RNA replicase in the presence of ATP markedly shortened the lag time for RNA synthesis, and ATP hydrolysis was accompanied by RNA synthesis due to the RNA replicase. The ATP hydrolysis was, however, not required for oligo(U)-primed initiation of RNA synthesis by the primer-dependent RNA polymerase.[182]

3. Role of VPg

Cap structures commonly found in eukaryotic mRNAs are absent at the 5′ terminus of poliovirus RNA.[183,184] Lee et al.[185] and Flanegan et al.[186] suggested that polioviral RNA was covalently linked to a small virus-specific protein, designated as VPg (genome-linked viral protein), at the 5′ terminus. The genomes of all picornaviruses carry VPg, although the length varies slightly in different viruses (for a review see Reference 151). This protein is not required for infectivity of the RNA.[187] After infection, VPg is removed from viral RNA by a cellular enzyme with a molecular weight of 27 kdaltons, which catalyzes the cleavage of the VPg-RNA linkage.[188,189] The presence of such an enzyme suggests that covalently linked RNA-protein complexes are present even in uninfected cells. Since the 5′ terminal

structure of plus-strand RNA was considered important as a signal(s) for the initiation of RNA synthesis, its translation or packaging, the role(s) of VPg has been studied extensively.

VPg is attached not only to the plus-strand RNA but also to the 5′ proximal poly(U) of the minus-strand RNA.[190,191] VPg is also detected with nascent RNA strands in the replicative intermediates,[187,190] suggesting that it is involved in the initiation of the synthesis of both minus- and plus-strand RNAs. The results of enzymatic and chemical analyses established that the linkage between VPg and both plus- and minus-strand RNAs is a phosphodiester bond between O^4 of tyrosine and the 5′ phosphate of uridylic acid.[192,193] VPg is the virus-coded protein 3B (see Figure 4); the nucleotide sequence coding for VPg is located within a region coding for polyprotein P3, which includes the RNA polymerase (3D) and the polyprotein-processing protease (3C).[194,195] VPg is composed of 22 amino acid residues and the RNA-linked tyrosine is located at the third position from the amino terminus.

Several single-strand RNA and double-strand DNA viruses contain proteins covalently linked to the 5′ termini of the viral genomes (for a review see Reference 196). For example, adenoviral DNA is covalently linked to the terminal protein (TP) at the 5′ terminus. During DNA replication, the precursor TP (pTP) is used as a primer and is covalently attached to the first nucleotide, dCMP.[197] The formation of pTP-dCMP is catalyzed by the viral DNA polymerase and enhanced by a host factor.[198] In the late stage of virus growth, the pTP is cleaved into the mature form, TP, by a viral protease. Accordingly, it is natural to consider that the poliovirus VPg is involved in the initiation of viral RNA synthesis, acting as a primer.

VPg has been found only as an RNA-associated form, i.e., not as a free form.[194,199] In analogy with the functional cycle of adenoviral TP, it was expected that a precursor for VPg exists as a free form and is able to bind to viral RNA. In accordance with this expectation, antibodies raised against synthetic polypeptides with the VPg sequence cross-reacted not only with the authentic VPg from virions but also with at least two or three proteins synthesized in virus-infected cells, all of which were found attached to RNA.[199-201] VPg is generated by processing a precursor polyprotein of the P3 region, which includes the gene for the RNA polymerase (see Figure 4). One of the processing intermediates contains the VPg sequence at its C terminus; its N-terminal region is hydrophobic in nature, suggesting that this precursor for VPg is membrane bound.[202] In fact, the membrane-associated RNA replicase was found to produce a genome-sized plus-strand RNA with VPg at its 5′ terminus; it is, however, inactivated by treatment with nonionic detergents.

Among the three assay systems for in vitro RNA synthesis by solubilized RNA polymerases, i.e., host factor-dependent viral RNA-directed RNA synthesis, oligo(U)-primed viral RNA-directed RNA synthesis, and oligo(U)-primed poly(A)-directed poly(U) synthesis, the anti-VPg antibodies were found to inhibit only the host factor-dependent RNA synthesis by the RNA replicase.[182,201,203] The immunoprecipitates contained the VPg precursor covalently linked to the newly synthesized RNA, which was 50 to 150 nucleotides in length and contained a large amount of poly(U) tracts. The VPg precursor(s) was covalently linked to RNA through a phosphodiester bond between tyrosine and UMP. These observations indicated that the VPg precursor(s), presumably contaminating the RNA replicase preparation, was involved in the initiation of host factor-dependent RNA synthesis. It is, however, not clear why the products linked to the precursor VPg were so short.

Supporting the hypothesis that VPg acts as a primer for RNA synthesis, a uridylated VPg, with the structure of VPg-pUpU, was isolated from infected cells.[204] This material was also synthesized in vitro using a membrane fraction of poliovirus-infected cells.[205] Recently, Takeda et al.[206] showed that a crude membrane fraction prepared from poliovirus-infected cells supported the synthesis of not only VPg-pU and VPg-pUpU, but also VPg linked to elongated RNA chains such as VPg-pUpUpApApApApCpApGp, which was detected among RNase T1 digests of elongated products and corresponded to the 5′-terminal oligonucleotide

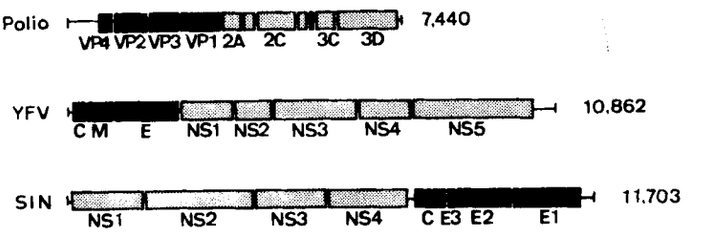

FIGURE 5. The genetic map of positive-strand RNA viruses. The complete sequences were determined for the genome RNAs of polioviruses of the Picornaviridae, yellow fever virus of the Flaviviridae, and sindbis virus of the Togaviridae. The genetic map of yellow fever virus is similar to that of poliovirus but is different from that of sindbis virus. Flaviviruses previously belonged to the Togaviridae and therefore are classified in a new family, the Flaviviridae.

of plus-strand RNA. In this system, the exogenously added VPg-pU could be elongated to VPg-pUpU. Furthermore, elongation was enhanced by the addition of an S-10 fraction prepared from uninfected cells. All of these observations indicated that the synthesis of plus-strand RNA is primed by VPg. This is in contrast with the initiation of the minus-strand RNA synthesis, which requires the host factor but does not necessarily depend on VPg. It cannot, however, be ruled out that VPg is attached to a yet unidentified primer for plus-strand RNA synthesis immediately after the initiation.

B. RNA Polymerases of Flaviviruses and Togaviruses

1. RNA Polymerase Genes of Flaviviruses

Until recently, flaviviruses were classified as a genus of the Togaviridae. In spite of general similarity with respect to the virion structure and the host range, however, there is a significant difference in the replication events between flaviviruses and alphaviruses. Furthermore, the sequence determination of the complete genomes of sindbis virus,[40] a prototype virus of the alphavirus subgroup of the Togaviridae, and of yellow fever virus[41] and West Nile virus[42,43] of the flavivirus group indicated that the gene organizations of the two viruses are completely different (Figure 5).

The genes encoding the structural proteins of yellow fever virus and West Nile virus are located at the 5′-proximal region of the genomes; all the genes are organized in a single long reading frame; no subgenomic mRNA is made. These characteristics are similar to those of the Picornaviridae. The complete sequence of Japanese encephalitis virus indicates essentially an identical gene organization.[434] Flaviviruses are therefore classified as a new family, the Flaviviridae.[207] The single- and plus-strand viral RNA contains a cap structure at the 5′ end but lacks a poly(A) tail at the 3′ end (for reviews see References 208 and 209). The flaviviral RNA is the largest mRNA that is completely translated in eukaryotic cells. In contrast with the case of picornavirus-infected cells, however, no full-length polyprotein has been detected in flavivirus-infected cells; it has therefore been considered that translation is initiated at multiple internal sites on a single RNA molecule.[210,211] Accordingly, the flaviviral RNA acts as a polycistronic mRNA, although translation initiation in eukaryotes generally takes place only at 5′ cap-proximal initiation codons. Such a phenomenon raised the hypothesis that many eukaryotic RNA viruses have prokaryotic characteristics (for a review see Reference 212). The posttranslational cleavage model of a single polyprotein cannot, however, be excluded yet. In fact, the presence of several precursor polyproteins was demonstrated in cells infected with either dengue 2 virus [213] or Kunjin virus.[214]

Among the major five nonstructural (NS) genes of flaviviruses (see Figure 5), the structural domains common to the largest subunits of RNA polymerases including the canonical GDD

sequence are present in the NS5 gene, i.e., the 3′-proximal gene (see Figure 3), implying that the NS5 protein is the catalytic subunit of RNA polymerase.

2. RNA Polymerase Genes of Togaviruses

The Togaviridae is a family of single- and plus-strand enveloped RNA viruses that replicate in the cytoplasm (for reviews see References 215 and 216). This virus family includes three genera, the alphaviruses, rubiviruses, and pestiviruses. The RNA genome is encapsidated by a single species of viral capsid protein and the nucleocapsid is enveloped by a lipid bilayer, in which viral RNA-coded glycoproteins are embedded. Most of our current knowledge on the transcription and replication of togaviruses comes from studies of two closely related alphaviruses, Semliki Forest virus (SFV) and Sindbis virus (SIN) (for reviews see References 217 and 218).

The complete sequence of the genome RNA is known for SIN,[40] which contains 11,703 nucleotides with a cap structure at the 5′ terminus and a poly(A) tail at the 3′ terminus. After infection, the viral RNA acts as an mRNA and produces two species of NS polyproteins, from which four NS proteins are generated by posttranslational processing (Figure 4). The first polyprotein contains the sequences of NS1, NS2, and NS3 and terminates at an opal (UGA) termination codon. The second polyprotein is produced by read-through of the opal termination codon and includes all the four NS proteins. This suggests the presence of natural suppressor tRNAs in eukaryotic cells. Genetic studies indicated that these NS proteins are involved in transcription and replication.[217,218] Temperature-sensitive SIN virus mutants defective in RNA synthesis at a nonpermissive temperature were grouped by a complementation assay into four groups. The role(s) of each NS protein is, however, not as yet known because the viral RNA polymerase has never been purified. The sequence comparison indicates that the canonical GDD sequence for viral RNA polymerase proteins[58,59] exists in the NS4 region (see Figure 3). One of the four NS proteins may be involved in the cleavage of the two species of NS polyproteins.

The newly synthesized RNA polymerase transcribes the infected plus-strand RNA into a full-length 49S minus-strand RNA, which subsequently serves as the template for the synthesis of two species of plus-strand transcripts, a full-length 49S RNA and a subgenomic 26S RNA. The genes for the structural proteins, a capsid protein (C) and three envelope glycoproteins (E1, E2, and E3), are organized in the 3′-proximal region of viral RNA and transcribed into the subgenomic 26S mRNA (Figure 4). In virus-infected cells, a temporal control operates for the synthesis of different RNA species and the translation of plus-strand RNAs. The viral capsid protein (C), which associates with RNA immediately after the synthesis, is expected to play a role in this regulation.

In early attempts to isolate an alphavirus replicase from infected cells, particulate preparations were obtained that synthesized double-strand RNA depending on an endogenous single-strand RNA, or both double-strand RNA and 49S and 26S single-strand RNAs (reviewed in References 217 to 219). Starting from these particulate fractions, soluble replicase complexes have been purified which contained the three NS proteins, NS1, NS2, and NS4, and had the ability to elongate RNA chains. The NS4 protein contains the consensus GDD sequence for RNA polymerase proteins from both plant and animal RNA viruses (see Figure 3). In addition, both NS1 and NS2 contain domains conserved between alphaviruses and certain plant viruses but not in flaviviruses (for a review see Reference 208).

Although togaviruses grow in the cytoplasm, the cell nucleus affects the rate and extent of virus growth. On this aspect, it is noteworthy that some incomplete SIN RNA species contain, at their 5′ termini, partial sequences identical to a cellular $tRNA^{Asp}$,[220,221] whereas their 3′-terminal sequences are identical to that of the parental infectious viral RNA. Such a tRNA species might serve as a primer for the virus RNA synthesis, as in the case of the influenza viral RNA synthesis (see Section V), but be removed at the end of polymerization.

IV. RNA-DEPENDENT RNA POLYMERASES OF NONSEGMENTED NEGATIVE-STRAND RNA VIRUSES

Negative-strand RNA viruses are classified into "group 5"[6] and comprise two families whose genome is a single uninterrupted RNA molecule, i.e., rhabdoviruses and paramyxoviruses, and three families with segmented genomes, i.e., arenaviruses with two RNAs, bunyaviruses with three RNA segments, and myxoviruses with eight RNA segments. These RNAs cannot serve as mRNAs in infected cells, and thus the naked RNA is insufficient to initiate an infection. All of these viruses contain helical nucleocapsids surrounded by a lipoprotein envelope which is acquired on budding from infected cells. Infection begins with the entry into the cells of the nucleocapsids, which contain a functional RNA polymerase. Nonionic detergents cause dissociation of the viral envelope that renders the core accessible to ribonucleoside triphosphates and allowing the RNA polymerase to synthesize RNA. This was demonstrated initially by Baltimore et al.[3] with VSV. Other rhabdoviruses also contain endogenous RNA polymerases.[222-224] Paramyxoviruses are similar to rhabdoviruses in many respects and contain RNA polymerases, although they are less active.[225-228]

Primary transcription of the infecting nucleocapsids produces the first mRNA molecules. Translation of these mRNAs produces viral proteins, including more RNA polymerase molecules, leading to amplified secondary transcription. Concomitantly, replication begins with the synthesis of full-length complementary RNAs (cRNAs), which in turn serve as templates for the synthesis of negative-strand genomic RNAs.

The 3′ and 5′ termini of the genomes of negative-strand viruses are roughly self-complementary and are considered to serve as recognition signals for viral RNA-dependent RNA polymerases and for encapsidation into virions. This structure provides a molecular basis for the mechanism through which single species of RNA polymerase can recognize and synthesize both plus- and minus-strand RNAs. The plus- and minus-strand nucleocapsids can be distinguished at the level of virion assembly. Most of this information came from studies on the molecular mechanisms of transcription and replication for VSV, a prototype of the Rhabdoviridae (for reviews see References 229 to 231). Previously, the classification of the Rhabdoviridae was based on the unique morphology of virions, but extensive molecular biological studies have shown that rhabdoviruses share many molecular characteristics, including the gene organization and the modes of transcription and replication.

A. RNA Polymerase of VSV

VSV is a bovine pathogen causing oral lesions and can be efficiently propagated in tissue culture. The genome is a linear single-strand RNA with negative polarity.[232] The viral envelope contains two viral-coded proteins, a glycoprotein (G) and a membrane (or matrix) protein (M). The G protein is located outside of the lipid bilayer and forms viral spikes, while the M protein interacts on one side with the envelope lining the inner surface of the lipid bilayer, and binds on its other side to a ribonucleoprotein (RNP) core to maintain the ordered helical structure. The RNP core contains the genome RNA, which is tightly complexed with approximately 2000 molecules of the nucleocapsid protein (N) and two other minor proteins, L and NS. NS was originally identified as a nonstructural protein but later found in virions as an essential subunit of viral RNA polymerase.[63,64] Thus we support a proposal that NS be designated as P, in analogy with the nomenclature of paramyxovirus genomes. Most of the N protein is spread throughout the RNP,[233] there being one N protein per five to six bases of RNA.

The viral RNA encodes all these five proteins. The primary structure of the genome RNA has been determined using cDNA cloning and DNA sequencing techniques.[45-47] The genome is 11,162 nucleotides long and consists of, from the 3′ terminus, the leader region, the five genes, in the order of N-P-M-G-L, and the 5′ terminal trailer region (Figure 6). The total

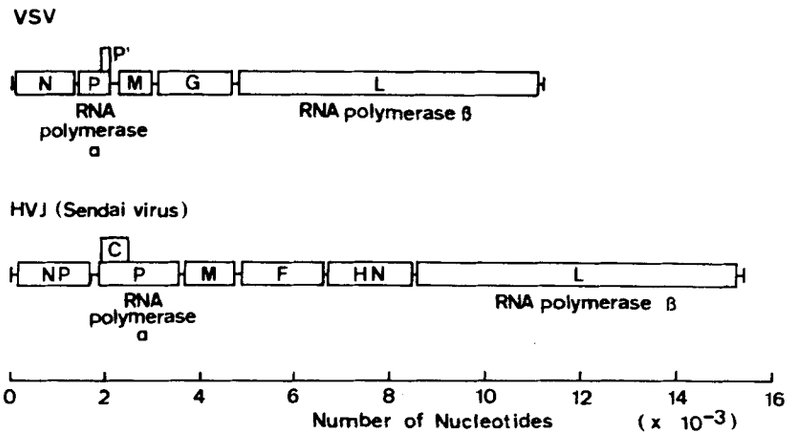

FIGURE 6. The genetic map of nonsegmented negative-strand RNA viruses. The complete sequences were determined for the genome RNAs of VSV, a prototype of the Rhabdoviridae, and HVJ (or Sendai virus), a prototype of the Paramyxoviridae. The genetic maps are similar between the two viruses except that HVJ contains an extra gene, F, which codes for the F protein with fusion activity. The P gene of VSV was originally identified as the NS gene encoding a nonstructural protein, NS. In this review, we propose to rename this gene as P, because the NS(P) protein is an essential component of the RNA polymerase in virions.

coding region is 93.9%, and the L gene alone accounts for 57.2% of the genome. In addition to these five protein genes, an open reading frame, designated as P′ in this review, was suggested, which overlaps the P gene in the same reading frame. Such an overlapping gene system in RNA genomes had been found in paramyxoviruses.[48] The C protein of HVJ is, however, coded for by a different reading frame from that of the P protein. An antibody raised against a synthetic peptide with a sequence corresponding to a part of this P′ protein was found to cross-react with a protein synthesized in VSV-infected cells.

1. Mechanism of RNA Transcription

The first step in the expression of these genes is their transcription from the parental nucleocapsid template by its intrinsic RNA polymerase. This transcription is independent of both host protein synthesis and host nuclear functions. The mRNAs are translated into the five viral proteins, including the RNA polymerase, which leads to accelerated synthesis of mRNAs, which is known as secondary transcription. Using purified virions and in the presence of an nonionic detergent and four ribonucleoside 5′-triphosphates, the five monocistronic mRNA species are synthesized in vitro sequentially, in the order of N-P-M-G-L,[234,235] indicating that virions conatin the RNA-dependent RNA polymerase as a structural component, which is able to transcribe the whole genome. Each mature mRNA contains an m^7GpppAm cap structure at the 5′ end[236] and a poly(A) tail at the 3′ end.[237,238] This indicates that the enzymes involved in mRNA modifications are also included in virions. In addition, a small leader RNA of 47 bases is synthesized, preceding the synthesis of N-mRNA.[239]

An alternative function of the negative-strand RNA-containing nucleocapsids is the synthesis of full-length positive-strand RNA (complementary RNA or cRNA), which serves as a template for the synthesis of progeny negative-strand RNA. RNA replication involves these two successive reactions. The virus life cycle involves a balance between the two alternate functions of the RNA polymerase, transcription and replication.[240-242]

mRNA transcription begins with the attachment of the RNA polymerase at the 3′ end of the genomic RNA; the enzyme migrates along the genome, synthesizing the leader RNA and five mRNAs in the sequential order. This was first indicated by an UV inactivation

Table 2
SUBUNIT STRUCTURES OF RNA-DEPENDENT RNA POLYMERASES

Virus	Subunit	Size	Proposed functions
VSV	α (P)	222	Template binding
	β (L)	2109	RNA synthesis, capping, poly(A) synthesis
HVJ	α (P)	701	Template binding
	β (L)	2226	RNA synthesis, capping, poly(A) synthesis
Influenza	(PB1)	757	RNA synthesis
	(PB2)	759	Cap binding
	(PA)	716	—

Note: The sizes of subunit polypeptides are shown in the number of amino acid residues. The proposed functions are generally based on several lines of indirect evidence; the functions of HVJ RNA polymerase subunits are estimated from the analyses on VSV and NDV RNA polymerases. For details see text.

map for transcription.[243,244] The sequential transcription is due to obligatory entrance of the RNA polymerase at the promoter located at the 3′ end of viral RNA.[245,246] Two controversial mechanisms have been proposed (reviewed in Reference 231): (1) the five genes are transcribed into a single polycistronic transcript, which is later processed by nucleases followed by RNA modifications (''RNA processing model''), and (2) transcription is initiated at the beginning of each gene and terminated at its end, thus leading to the production of monocistronic transcripts (''termination and reinitiation model''). Poly(A) tails of individual mRNAs are believed to be generated by repeated transcription of a stretch of seven U residues located between genes. The first model implies that the RNA polymerase carries the activities of the endonucleolytic cleavage of polycistronic read-through transcripts and the addition of a cap structure at the 5′ end of each cleaved product. In contrast, the second model implies that the RNA polymerase terminates transcription after the poly(A) synthesis and reinitiates the transcription of downstream genes, the cap structure being added to each of the newly initiated transcripts.

2. Subunit Structure and Multiple Functions

All of the three core-associated proteins, N, L, and P, are absolutely required for the viral transcription and replication. The major protein species in RNP is N, which tightly associates with RNA to form RNase-resistant structures, which cannot be dissociated even on CsCl centrifugation.[64] The large size and low abundance of L has indicated that this protein is responsible for the catalytic functions of polymerization, capping, methylation, and polyadenylation. Both the L and P proteins were individually inactive, but the reconstitution of mRNA and leader RNA synthesis was achieved only when both the L and P proteins were added to the N-RNA complex.[63,64] It was therefore concluded that both the L and P proteins are essential subunits of the RNA polymerase holoenzyme. Deproteinized RNA is not infectious but RNP containing the L and P proteins is infectious provided that DEAE-dextran is added to promote cellular uptake.[247] In analogy to the well-characterized bacterial RNA polymerases (for a review see Reference 248), we propose to designate the P and L proteins as the α and β subunits, respectively, the catalytic site being located on the β subunit (Table 2). A 1:1 molar ratio of the L (β subunit) and P (α subunit) proteins is required for RNA synthesis in vitro.[63,64] Monospecific antisera against the L and P proteins inhibited RNA synthesis by RNP in vitro.[249,250] The results of a detailed study suggested that β subunit (L) alone is capable of synthesizing oligonucleotides constituting the 5′ termini

of the leader RNA and mRNA sequences.[251] At saturating concentrations of the β subunit (L), all RNA chains are rapidly started, whereas the chain elongation is rate limiting, due to the limited concentration of the α subunit (P).[252] On the other hand, when the α subunit (P) is in excess, the rate-limiting step is the initiation of RNA synthesis by the β subunit (L). Thus, all the initiated RNA chains are quickly extended and completed in the presence of excess α subunit (P). In virus-infected cells, the P protein is synthesized in considerable excess over the amount which is ultimately incorporated into virions, and a substantial fraction remains free in the cytoplasm.[253] Several lines of evidence supported the notion that the αβ complex catalyzes not only RNA synthesis but also capping,[254] methylation,[255] and polyadenylation.[256] None of these activities could be separated from the complex. Since exogenous RNAs could not be utilized as substrates for any of the modification functions, it was considered that the enzyme complex required nascent transcripts as substrates for the RNA modifications. From these studies, it has been envisaged that the template for RNA synthesis is the N-RNA complex, whereas the soluble proteins L(β) and P(α) constitute the RNA polymerase holoenzyme (αβ complex) and catalyze both the transcription of the template and the modification of transcripts leading to the formation of the five monocistronic mRNAs and the leader RNA. The two complementing mutants of the New Jersey serotype of VSV have *ts* lesions in the L protein,[257] indicating that the L protein has at least two independently mutable functions. Reconstitution experiments indicated that the catalytic sites for all these enzymic activities are located on the L protein.[258,435]

The M protein appears to play a role in the regulation of transcription as well as morphogenesis. Crude virus fractions containing the G and M proteins inhibited RNA synthesis in vitro only at low ionic strength.[259] Also, nucleocapsids retaining the M protein were transcriptionally inactive at low ionic strength; the level of transcription inhibition increased on the addition of purified M protein.[260] The inhibition of RNA synthesis occurred at the level of RNA chain elongation and not at the initiation site.[260] Thus, it appears that the M protein binds to RNP and prevents the migration of RNA polymerase.[261] The interaction of M with RNP possibly occurs at the membrane prior to or at the same time as the budding process. This interaction might cause RNP to cease RNA synthesis prior to virus maturation. Accordingly, it is reasonable to assume that the RNA polymerase activity appears when the M protein is removed from RNP during the uncoating after virus infection. The domain(s) of the M protein responsible for the interaction with lipid bilayers and with RNP are being examined using monoclonal antibodies[262] and by means of protein chemistry.

3. Mechanism of RNA Replication

The RNA polymerase (L-P complex) has two activities, i.e., transcription and replication, and exists in two functionally distinct forms. In vivo, RNA replication begins about 1 hr after infection and requires the continued synthesis of viral proteins. The synthesis of the full-length complementary RNA (cRNA), a required intermediate for replication, has been detected only under certain in vitro conditions.[263-265] Using a transcription-translation coupled system, it was found that the N protein is required for the synthesis of cRNA by read-through transcription,[266,267] which is initiated from the extreme end of the genome RNA, but the synthesis of new RNA polymerase is not required for replication.[268] Thus, the RNA replication is N protein dependent and only encapsidated RNAs (RNA-N protein complexes) are produced. The following model has been proposed: the N protein associates with nascent RNA, thereby allowing read-through transcription to the 5′ end of the template RNA.[269-271] In fact, VSV mutants with mutations in the N protein gene produce altered N proteins, which suppress the termination at leader RNA sites.[272] Substitution of ITP for GTP facilitates this read-through transcription and results in the synthesis of cRNA without N protein synthesis,[273] suggesting that either an inter- or intramolecular base-base interaction is involved in the read-through transcription.

Phosphorylation and dephosphorylation of P protein play a key role in the regulation of transcription and replication. Evidence supporting this model has accumulated: dephosphorylation in vitro of P by phosphatases results in a reduction in the transcription activity of nucleocapsid-bound RNA polymerases,[274] inhibitors of the P phosphorylation inhibit in vitro RNA synthesis,[275-277] and only the most highly phosphorylated P fully restores the RNA polymerase activity when combined with isolated L protein.[278] All these observations together indicated a direct interaction of L-P and its modulation through the phosphorylation of P.

The second function of P is to bind free N. Interactions between N and phosphorylated P could affect two processes: (1) P displaces tightly bound N from the template in a localized manner, thereby allowing the RNA polymerase to gain access to the template RNA; and (2) P forms complexes with N in the cytoplasm,[279-281] preventing the self-aggregation of unassembled N. As noted above, the availability of unassembled N regulates the switching from transcription to replication.[269-271] Thus, a mechanism for maintaining N in a soluble form is required for RNA replication. In fact, multiple forms of the N-P complex, the putative storage form of the N protein, were detected, which differ in their sedimentation properties and N to P ratios.[280]

The P and M proteins are the major phosphoproteins in VSV infected cells.[282-283] P is an exceptional protein as to the extent of phosphorylation. Its primary sequence contains 12 threonine and 21 serine residues, i.e., a total of 33 potential sites of phosphorylation.[281-284] The P protein is phosphorylated in vivo and in vitro on various subsets of as many as 21 serine and threonine residues, which reside mostly in the N-terminal third of the molecule.[284] Phosphorylation of tyrosine residues has also been suggested.[285] A heavily phosphorylated, extremely acidic domain near the N terminus of P constitutes a novel structure that resembles the phosphate backbone of RNA and is, therefore, capable of binding the N protein.[281] Phosphorylated P migrates as two discrete electrophoretic species on polyacrylamide gels in the presence of both SDS and urea; the highly phosphorylated form of P, P2, migrates faster than the less phosphorylated form, P1.[284] P2 differs from P1 in the possession of additional phosphorylated residues.[284-288] P1 could be converted to P2 by phosphorylation in vitro and the conversion of P2 to P1 could be performed in vitro with phosphatases.[284] The difference in the phosphorylation level might affect the binding affinity of P to the N protein. Deletion mapping using cDNA clones indicated that a specific domain between amino acid residues 213 and 247 was essential for transcription, but the C-terminal 21 amino acids with a highly conserved sequence between different serotype viruses can be deleted without significant effect on transcription.[289] These experiments may contribute to the detailed mapping of functional domains on the RNA polymerase proteins.

Both the N and P proteins were phosphorylated in vitro when purified virions were incubated in the presence of a nonionic detergent and [$\gamma - {}^{32}$P]ATP. This indicated that a protein kinase activity was associated with the virions.[282,283,290] Sanchez et al.[290] reported that the isolated L protein specifically phosphorylated the P protein in vitro. Exogenous acceptors such as phosvitin and casein were also phosphorylated by the L protein. However, both cellular and viral protein kinases seemed to be involved in the P phosphorylation because cellular protein kinase(s) phosphorylated P2 in the presence of the N-RNA complex.[291] Phosphorylated P2 is susceptible to the action of cellular phosphatases.

Progeny viral RNA (vRNA) with minus-polarity is synthesized by transcribing cRNA (full-length plus-strand RNA). Some yet unidentified mechanism must exist for temporal control over the synthesis of cRNA and vRNA, because cRNA is the major species early in infection, while vRNA is predominant later.[292,293]

4. Defective-Interfering (DI) RNA

The phenomenon of multiplicity-dependent production of noninfectious virus particles, known as the "von Magnus phenomenon", has been observed in almost all animal viruses,

and the term "defective-interfering" (DI) particles was introduced for the incomplete noninfectious virus particles exhibiting interfering activity with the multiplication of complete standard infectious viruses (for reviews see References 294 to 297). DI-mediated interference occurs intracellularly and not at the cell surface receptor; it is not attributed to interferon, and the interference is strongest for the standard virus from which the DI particles were derived. A number of different DI genomes have since been studied at the level of the DNA and RNA sequencing. Among the best studied are the DI RNAs of VSV (reviewed in References 294 to 297). These studies clearly indicate that all DI viruses are noninfectious because of deletions in their genomes, but can still multiplicate in the presence of the helper function of complete viruses. Therefore, this is a good model system for elucidating the unique characteristics of replicating RNA molecules.

A large number of DI particles of VSV have been identified that contain RNAs of different lengths and from different regions of the parental genome. The same progenitor gene can give rise to multiple DI RNAs of varying length. Four classes of DI particles have been identified: deletion, snapback, panhandle, and compound DI particles.[298-300] Despite their structural diversity, two common characteristics of all DI particle genomes are that part of the RNA polymerase gene (L) is deleted and that their replication is dependent on the RNA polymerase provided by the helper virus. Their 5′ termini have minus polarity and are identical to those found on the parental genome, and their 3′ termini are also highly conserved and are either identical to the 3′ termini of the minus-strand genome RNA or totally complementary to the 5′ termini of plus-strand RNA. The conserved sequence is believed to be the recognition signal for the single species of VSV RNA polymerase holoenzyme.

The results of sequence analysis of DI RNAs [301,302] supported the copy choice model, which suggests that the RNA polymerase prematurely ceases RNA synthesis and moves with the nascent daughter strand to another site on the same template molecule, where elongation of the RNA chain is resumed. This mechanism produces a copy-back type molecule. The RNA polymerase-nascent RNA complex might also move to a different molecule to produce a mosaic RNA.

B. RNA Polymerases of Paramyxoviruses

The Paramyxoviridae is a family of negative-strand RNA animal viruses containing a single molecule of RNA as their genome (for reviews see References 303 to 305). Transcription and replication have been studied for Newcastle disease virus (NDV) or hemagglutination virus of Japan (HVJ), known as Sendai virus. The genome RNA is present in a helical nucleocapsid containing the RNA polymerase. The capsid is enveloped by a cell-derived lipid bilayer containing two glycoproteins (HN and F) and an internal nonglycosylated polypeptide (M). HN is the surface glycoprotein found in spikes outside the lipid bilayer and is equivalent to the G protein of VSV. The HN glycoprotein of paramxoyviruses, however, plays two functions: it acts as both hemagglutinin and neuraminidase. The F protein is unique to paramyxoviruses and has no counterpart in rhabdoviruses; it is the protein that controls fusion and hemolysis. As in the case of VSV, the M protein is crucial for virus assembly and the budding of mature virions; it may also be involved in the control of the expression of RNA polymerase activity during virus infection and can be phosphorylated in vivo and in vitro.

Paramyxovirus infections share many common characteristics with that of rhabdoviruses. Transcription, translation, and replication take place in the cytoplasm and are independent of nuclear functions; these viruses grow in UV-irradiated cells, in the presence of either α-amanitin or actinomycin D, and in the presence of inhibitors for cellular DNA synthesis. In contrast to rhabdoviruses, however, no infectious viruses are produced in enucleated cells; the nucleus seems to be required for the final maturation of paramyxoviruses. Primary transcription by the virion-associated RNA polymerase leads to the formation of at least six

known species of capped and polyadenylated monocistronic mRNAs, even in the absence of protein synthesis. Replication of the genome and secondary transcription both require protein synthesis.

Endogenous RNA polymerases were identified using detergent-disrupted virions of NDV,[225] HVJ,[226,227] and mumps virus.[228] In addition to the synthesis of capped and methylated monocistronic mRNAs, the first step of replication leading to the formation of full-length plus-strand RNA (cRNA) takes place under certain conditions. The active transcription complex is nucleocapsids, containing one molecule of RNA, approximately 2500 molecules of the nucleocapsid protein (NP) about 100 to 300 copies of the P protein with a molecular weight of 53 kdaltons, and about 30 to 60 molecules of the L protein with a molecular weight of 200 kdaltons. Transcription starts at a single promoter in vivo and the order of the genes has been determined from the results of UV transcriptional mapping to be 3′-NP-P-M-F-HN-L-5′ (Figure 6). This gene order has been confirmed by sequence analysis of the entire genome of HVJ (or Sendai virus).

The structures and functions of RNA polymerases of paramyxoviruses have been studied for NDV, HVJ (or Sendai virus), and SV5. The functional holoenzyme was found to be composed of two polypeptides, L and P[306,307] (Table 2). This was directly confirmed by the results of a reconstitution experiment on active transcriptive complexes formed from the isolated L and P proteins.[69] Thus, the P protein corresponds, in both structural and functional respects, to the P(NS) protein of VSV. The P and L proteins should therefore be designated as the α and β subunits, respectively. As has been found in VSV, the α subunit (P protein) exists in multiple phosphorylated forms and may play some role in the regulation of RNA polymerase activity.

Several lines of evidence suggested that the synthesis and assembly of viral ribonucleoprotein cores take place in association with the cellular cytoskeletal framework.[308,309] For example, the newly formed RNP core of NDV was isolated as a complex with the cytoskeleton and exhibited viral RNA synthesis on the framework.[309] In this context, the addition of cellular factors has been shown to stimulate transcription in vitro by detergent-disrupted HVJ and VSV.[310] Recently, Moyer et al.[73] reported that tubulin is a host factor because the addition of purified tubulin stimulates in vitro viral RNA synthesis, both monoclonal and polyclonal anti-tubulin inhibit the reaction, and antibodies coprecipitate viral RNA polymerase along with tubulin. Likewise, Hill et al.[74] found the involvement of microtubule-associated protein (MAPs) for in vitro transcription and replication of HVJ. Like VSV, DI genomes of HVJ are generated by a copy-choice mechanism,[311] involving the termination, the RNA polymerase jumping to other recognition signals on the same or different RNA molecules, and the reinitiation of RNA synthesis.

V. RNA-DEPENDENT RNA POLYMERASES OF SEGMENTED NEGATIVE-STRAND RNA VIRUSES

Influenza viruses belong to "group 5".[6] There are three different types: A, B, and C, of which the A viruses cause the most severe diseases in humans. Influenza A viruses share an immunologically related internal nucleocapsid protein (NP), which can be regarded as group-specific antigen different from those of influenza B and C viruses. The internal antigen provides the molecular basis for their classification into types. Typing of influenza A and B viruses on the basis of the matrix protein (M) is consistent with that on the base of NP. The influenza A viruses are further subdivided on the basis of antigenic differences in their two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). According to the present nomenclature system proposed by the World Health Organization (WHO) in 1980,[312] the H antigens are grouped into 12 subtypes and the N antigens into 9 subtypes.

The viruses consist of approximately 1% RNA, 5 to 7% carbohydrate, 18 to 37% lipid,

and 60 to 70% protein. The surface spikes of virions contain two different virus-coded glycoproteins, HA and NA, in a lipid bilayer coded for by the host cells. The inner core of the virions is surrounded by the matrix (M) protein and contains RNA segments that are associated with the nucleoprotein (NP) and three different subunits of RNA polymerase, two basic (PB1 and PB2) and one acidic subunit (PA).

The observed high rates of genetic recombination after co-infection of cells with genetically distinct influenza viruses led to the proposal that influenza viruses possess a segmented RNA genome[313] and that the frequent exchange of genetic information reflects the reassortment of individual RNA segments.[314] This concept was supported by biochemical characterization of viral RNA. PAGE clearly showed that the influenza viral genome is composed of segmented RNAs.[315,316]

Antigenic variation of influenza viruses is the result of genetic changes in the surface proteins, HA and NA. Two different types of natural antigenic variation occur, antigenic "shift" and antigenic "drift". After long intervals of about 10 to 20 years, an antigenically completely new influenza subtype appears to which the population has no immunity. This type of antigenic variation is called an "antigenic shift" and involves reassortment of RNA segments coding for either HA or NA proteins. Thus, the segmented nature of the genome plays an important role in the epidemiology of influenza viruses, particularly as to the origin of the pandemic strains for humans. On the other hand, an "antigenic drift" involves a series of minor and gradual changes in the surface protein genes of a subtype, resulting from the selection by an immune host population of mutant virus particles.

A. RNA Polymerase of Influenza Virus

Several lines of evidence indicated that influenza viral RNA is negative stranded: (1) RNA alone is not infectious; (2) virions contain an RNA-dependent RNA polymerase activity; (3) purified RNA from infected cell polysomes completely protects virion RNA after hybridization; (4) RNA from infected polysomes directs the synthesis of virus-specific proteins in eukaryotic cell-free translation systems, whereas RNA from purified virions does not; and (5) only RNAs from polysomes, i.e., not virion RNAs, contain a cap-structure at the 5′ termini and poly(A) at the 3′ termini (Reference 319 and references therein).

The negative-strand RNA genome is divided into eight segments,[317,318] and the gene products of each segment have been characterized to provide a complete genetic map (Figure 7).[319-321] On PAGE, however, different influenza viruses show characteristic differences in the migration rates of the eight RNA segments.[318,319] Each of the six large RNA segments is transcribed into a monocistronic mRNA coding for one of the viral proteins, whereas the two small segments are transcribed into single capped and polyadenylated RNAs coding for M1 and NS1, respectively, some of which are, however, processed to generate spliced mRNAs coding for M2 (and M3) and NS2, respectively.[322-324] Thus, there are sequences which act as exons in the primary transcripts but as introns for the generation of secondary (or tertially) transcripts. RNA viruses employ such overlapping gene systems to increase the content of genetic information without concomitant increase of the genome size.

In A/PR/8/34(H0N1), the three largest segments code for three subunits (PB1, PB2, and PA) of the RNA polymerase. RNA segment 4 codes for the HA, 5 for the NP, 6 for the NA, 7 for the matrix (M) proteins, and 8 for the NS proteins.

1. Mechanism of RNA Transcription

Two types of transcript are produced in influenza virus-infected cells (Figure 8). One type (mRNA), which consists of incomplete transcripts of the genome RNAs, is capped and polyadenylated, becomes associated with cell polysomes and then directs the synthesis of viral proteins; the other type (cRNA) comprises full-length transcripts without a cap structure or poly(A) tail, which act as the templates for RNA replication. There are two main aspects

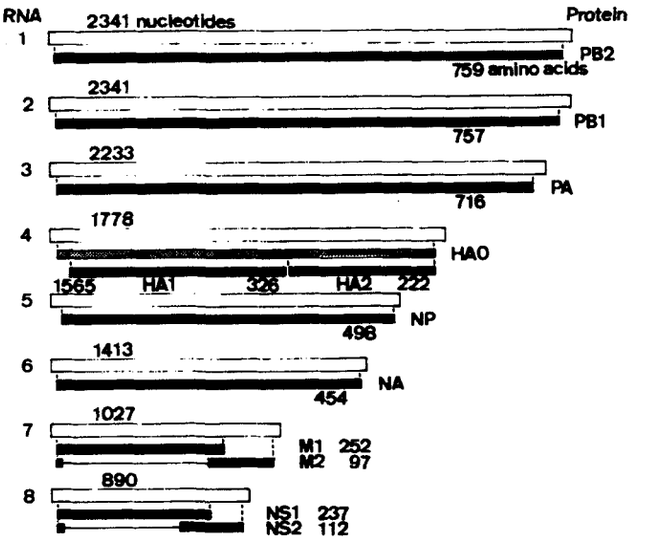

FIGURE 7. The genetic map of influenza virus. The complete sequences of all eight RNA segments have been determined for the influenza virus A/PR/8/34. Each RNA segment codes for a single species of protein except the small two segments, RNA 7 and RNA 8, which encode additional proteins, M2 and NS2, after splicing of the respective primary transcripts.

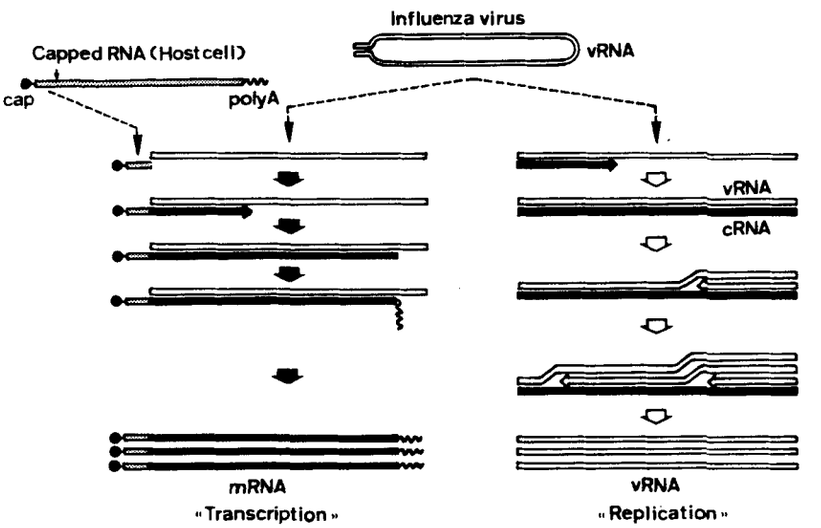

FIGURE 8. Transcription and replication of the infleunza viral genome. The influenza viral RNA polymerase catalyzes the cleavage of capped RNA in host cells and, using the resulting capped fragments as primers, synthesizes viral mRNA. The addition of a poly(A) tail is also catalyzed by the viral RNA polymerase. For the replication of viral RNA, the RNA polymerase seems to be modulated to a form, which is able to synthesize a full-sized cRNA without these modifications. cRNA directs the synthesis of vRNA.

of the control of influenza virus transcription: (1) regulation of the synthesis of individual mRNAs and (2) the differential production of the two classes of transcripts, i.e., mRNA and cRNA (complementary RNA or template RNA for replication).

In sharp contrast to rhabdoviruses and paramyxoviruses, influenza viruses have been recognized to be unique among nononcogenic RNA viruses because host nucleoplasmic RNA synthesis is continuously required for viral transcription to occur. Primary transcription is blocked by the addition of α-amanitin, a potent inhibitor of host RNA polymerase II, and takes place when α-amanitin-resistant mutant cells are used.[325,236] A similar effect was seen with treatments that block the function of DNA but not of the RNA polymerase itself.[327,328]

A clue explaining the requirement for the host cell RNA polymerase II function was the finding that the addition of dinucleotides stimulated influenza virus-associated RNA polymerase activity[329] and that the added dinucleotides were incorporated at the 5′ ends of newly synthesized RNA chains. The most efficient primer was either ApG or GpC.[329,330] Essentially, the same dinucleotide primer requirement spectrum was found on more systematic and quantitative analysis, in which the dinucleotide-dependent formation of trinucleotides was measured for dinucleotides of various sequence combinations.[331] In the absence of dinucleotide primers, efficient transcription was found to take place in reticulocyte lysates.[332] This finding led Krug and colleagues to propose that the intracellular primer is not a dinucleotide but rather an RNA synthesized by RNA polymerase II. One piece of direct evidence was that globin mRNA and other cellular mRNAs stimulated the transcription.[332] The stimulation by globin mRNA was about 1000 times greater than ApG. Furthermore, 5′-terminal sequences of 10 to 15 nucleotides in length of globin mRNA including the cap structure were found in the viral transcripts.[333] Both the 7-methyl on the terminal G and the 2′-*O*-methyl on the penultimate base (the cap-1 structure) were needed for the priming activity.[334] The absolute requirement of the cap-1 structure was confirmed by the endonucleolytic cleavage assay of capped RNA.[335] This is the first instance in which the cap-1 structure was found to have a definite effect on a specific function of an mRNA.

This stimulation of transcription in vitro by mRNAs was considered to reflect a requirement for similar molecules by virus-specific RNA polymerases in vivo. In fact, direct analyses of the 5′ termini of in vivo RNA indicated that nonviral sequences of similar length were present in viral mRNA molecules.[336-338] All these observations together indicated that the synthesis of viral mRNA requires primers generated from cap-1 (m^7GpppNm)-containing RNAs; both hnRNA and mRNA transcribed by RNA polymerase II are possible candidates for these primers. However, snRNAs transcribed by RNA polymerase III seem not to be primers because RNAs with a trimethylated cap structure such as snRNAs did not stimulate RNA synthesis in vitro by influenza viral RNA polymerase.[436]

Besides the priming function of viral transcription, the cap-1 structure activates the influenza viral RNA polymerase by allosteric modulation, because free cap-1 monomers, i.e., m^7GpppNm not associated with RNA, stimulated the viral RNA synthesis and this stimulation was additive with that by dinucleotide primers.[340,341] This is a novel physiological function found for the cap-1 structure.

The first step of capped RNA-primed transcription is the cleavage of capped RNA by a capped RNA-specific endonuclease associated with the viral RNA polymerase. On the bases of the results of sequence analysis of the primer-transcript junction region of several viral mRNA species, Krug and colleagues proposed that the endonuclease preferentially cleaves next to purine residues.[339] However, the enzyme cleaves capped poly(U) as well.[335] To account for all these observations, we proposed that it cleaves phosphodiester bonds at the 5′ side of either A or U residues.[335] The cap-1 structure, i.e., a cap structure containing both the 7-methyl on the terminal G and 2′-*O*-methyl on the penultimate base, is absolutely required for the cleavage of capped RNA.[335] mRNA synthesis is initiated by the addition of a G residue that is complementary to the second nucleotide to the 3′ end of viral RNA

segments.[335,339] Thus, primers need not be hydrogen bonded with viral RNA templates. The viral mRNA chains are then elongated.

As to the termination of transcription, two mechanisms have been proposed which would lead to the generation of incomplete transcripts carrying a poly(A) tail.[342,343] Transcription leads to the formation of complete transcripts, some of which are subsequently nucleolytically processed before polyadenylation. Alternatively, RNA synthesis terminates before transcription of the 5′-terminal regions of the genome segments, with a switch to poly(A) synthesis. In the absence of protein synthesis, most of the transcription produces only mRNA, suggesting that premature termination is the most likely mechanism and that the newly synthesized proteins are required for antitermination. The tract of 6 U residues between position 17 and 22 from the 5′ termini might represent a termination signal which is similar to the termination signal of prokaryotic transcripts.

During influenza virus growth, two phases of transcription can be distinguished which are designated as "primary" and "secondary" transcription. Primary transcription is catalyzed by the action of an enzyme, which is an integral component of infectious virus particles, and occurs independently of protein synthesis. Secondary transcription depends on the continuous synthesis of viral proteins, representing predominantly the transcription of progeny virus RNA molecules. In the presence of cycloheximide, transcription is restricted to the action of the RNA polymerase of the infecting virus particles, which leads to the production of mRNA. This primary transcription process is selective,[344] presumably due to the uneven distribution of the RNA polymerase among RNA segments. Virions contain 10 to 20 molecules of the RNA polymerase, which seem to be unequally distributed among eight RNA segments.[345] The preferential synthesis of certain mRNAs may therefore be the result of selective binding of the RNA polymerase. In this regard, the conserved sequences of the first 12 nucleotides at the 3′ termini and the first 13 nucleotides at the 5′ termini of all the RNA segments are believed to form the signal for RNA polymerase binding. The association of the RNA polymerase with RNA termini has indeed been observed with isolated nucleocapsid cores and the RNA polymerase-RNA complexes from influenza virus PR8.[345] These observations, however, suggest that RNA provides no structural basis for the selective binding of the RNA polymerase. Restricted primary transcription is also a reflection of an undefined interaction between viruses and cells, because the initial transcripts vary depending on the host cells.

Newly synthesized proteins specified either by the host or the virus are required for the production of cRNA (or template RNA for vRNA synthesis). There are two possibilities: (1) the input RNA polymerase is modified by newly synthesized proteins, and (2) the newly synthesized RNA polymerase in infected cells is structurally and functionally different from the input polymerase. The virus-specific RNA polymerase is induced between 1 and 2 hr after influenza virus infection, in both the microsomal and nuclear fractions.[346] The microsomal enzyme synthesizes only plus-strand RNA in vitro,[347] and the nuclear enzyme synthesizes both plus- and minus-strand RNAs.[348-351]

With respect to secondary transcription, a regulation mechanism operates which leads to the preferential synthesis of certain mRNAs. The time of the maximum rate for individual mRNA synthesis is different and the host cells may influence this control.

2. *Subunit Structure and Multiple Functions*

Following solubilization of the virus membrane with a nonionic detergent, purified virions of influenza viruses exhibit RNA-synthesizing activity in the presence of four ribonucleoside 5′-triphosphates,[352-354] indicating that the viral RNA polymerase is an intrinsic component of viral cores. Isolated nucleocapsid cores are also active in RNA synthesis.[67,345,353,355] Four virus-specific proteins are associated with the cores: the nucleocapsid protein (NP), which represents over 90% of the total core proteins, and three P proteins which are separated on

two-dimensional gel electrophoresis into two basic, PB1 and PB2, and one acidic protein, PA.[356] The cleavage of capped RNA, primer-dependent initiation of RNA synthesis, elongation of RNA chains, and termination and polyadenylation of the transcripts are all carried out by these complexes. As described later, however, RNA replication, i.e., the minus-strand RNA-dependent synthesis of full-sized plus-strand RNA without modifications (cRNA) and cRNA-dependent synthesis of minus-strand RNA (vRNA), requires a yet unidentified additional factor(s).

DNA replication involves an associated error-suppressing and proofreading mechanism (reviewed in Reference 357). This is attributed to an editing function of DNA polymerases, i.e., mismatched bases in growing DNA molecules are excised and correct bases are recopied. In contrast, the high error rate of RNA replication has been explained as due to the lack of an error-correcting function of RNA replicases. However, we have recently found that the influenza viral RNA polymerase is capable of replacing bases at the growing ends of nascent RNA molecules.[359] This apparent proofreading activity may result in the insertion of mismatched bases into genome RNAs, leading to the generation of variant viruses. Genome segmentation, a characteristic of certain RNA genomes, including influenza viruses, is thought to be an evolutionary means of keeping the identity of the genome within the fidelity limits of the RNA replicases. The genome segmentation also provides a means of reassorting RNAs from different sources and hence allows the generation of variant viruses with genome substitutions. The results of genetic studies indicated that both PB1[360-363] and PB2[361,362,364,365] are essential for primary transcription; in addition, the replication requires PA as well.[366-368] In addition to the three P proteins, NP is required for replication.[364,365,369] An NS protein, which is present only in virus-infected cells, is also involved in RNA replication.[370-372] Various attempts have been made to establish the functions of individual proteins. The results of UV-induced cross-linking experiments indicated that PB2 was involved in the recognition and binding of the cap structure, whereas PB1 was involved in the initiation and elongation of RNA chains[373] (see Table 2). It is, however, not yet known which P protein contains the catalytic sites for capped RNA cleavage and phosphodiester bond formation.

Centrifugation of RNP cores in either CsCl or CsTFA leads to dissociation of NP, leaving P proteins bound to RNA;[67,68,345] this is in sharp contrast with VSV cores, from which the RNA polymerase (P-L complex) can be dissociated before the N protein on CsCl centrifugation.[64] The isolated RNA-P protein complexes devoid of NP were found to be active in RNA synthesis. However, transcription of NP-free RNA by the P protein complex ceases soon after transcription initiation.[68] Np is, therefore, required for the elongation of RNA chains. After dissociation of P proteins from RNA-P protein complexes, all three P proteins are still associated,[68,437] indicating that the RNA polymerase holoenzyme is a complex containing one molecule each of the three P proteins. By adding viral RNA, the P protein complex regains an RNA-synthesizing activity.[438] This supports the model implying that the P protein complex moves down viral RNA templates together with elongation of RNA chains.

As in VSV, the influenza viral M protein associates with RNP and regulates the function of RNA polymerase.[67,71,439] Genetic studies also indicated synergism between the M and P protein genes.[374] These observations support the model that during virus maturation, the M protein prevents the RNA polymerase from working, but during infection, the RNA polymerase starts working concomitantly with uncoating of the M protein.

In virions, the RNA polymerase seems to be located at the 3′ end of viral RNA segments: (1) transcription initiates at 3′ ends of viral RNA;[331] (2) the RNA polymerase prevents 3′ labeling of viral RNA with [$\gamma-{}^{32}$P]pCp and RNA ligase;[345] (3) the 3′-terminal region of RNA-RNA polymerase complexes are protected from hydrolysis by nucleases;[345] (4) a consensus sequence exists at the 3′ termini of all eight segments,[375,376] which is believed to be a signal for the binding of a single species of RNA polymerase; and (5) all RNA molecules contained in DI particles retain the 3′-terminal consensus sequence.[377]

3. Mechanism of RNA Replication

Another plus-strand transcript (cRNA) species lacks both the cap structure and the poly(A) tail, the 5′ terminus being pppA and the 3′ terminus being the complete transcript down to the 5′ end of the template RNA. The synthesis of cRNA is dependent on the continued production of functional viral proteins, which modify the function of the RNA polymerase as follows: (1) transcription by the modified RNA polymerase can be initiated without primers and (2) transcription elongation proceeds beyond the tract of six U residues known as the putative polyadenylation signal. The cRNA thus produced can direct the synthesis of minus-strand RNA (vRNA). This step is also dependent on viral protein synthesis.

In vitro systems for RNA replication have been developed by three groups[349-351] using crude extracts or isolated nuclei from influenza virus-infected cells. In these systems, not only mRNA but also cRNA and vRNA were synthesized to various extents. Under the same reaction conditions, the virus-associated RNA polymerase was unable to support the RNA replication. Thus, influenza-infected cells must contain a factor(s) that affects the specificity of the RNA polymerase. Preliminary fractionation of a nuclear extract of infected cells separated the putative replication factor that affects the specificity of the RNA polymerase from the primer-dependent mRNA-synthesizing activity.[378] Another unique characteristic of our in vitro replication system is the requirement for high concentrations of ATP,[351] which might reflect the *de novo* initiation of RNA synthesis.

Several lines of evidence have indicated that the synthesis rates for the three different classes of viral RNAs, i.e., mRNA, cRNA, and vRNA, for each RNA segment are controlled throughout infection. Immediately after infection, mRNA is the predominant species and the rate of its synthesis reaches a peak at 2 to 3 hr of infection; afterwards, both cRNA and vRNA increase in parallel.[379-382] The in vivo kinetics of the appearances of the three classes of RNA indicated that a factor(s) involved in the switching of the specificity of the RNA polymerase is induced within a few hours after infection; this induction is blocked by the addition of protein synthesis inhibitors.[380,383] Viruses with temperature-sensitive mutations in RNA segment 8 are more or less defective in RNA replication.[370-372] The results of detailed analyses indicated that the switch from early to late protein synthesis does not occur in these mutants. The NS proteins are associated with the nuclei of infected cells.[384-386]

The syntheses of mRNAs and proteins are under temporal control.[382,383] The relative level of each mRNA and protein species is controlled; during the early phase of infection, mRNAs coding for NP and NS predominate, whereas mRNAs for NA, HA, and M increase at a later stage. The differential transcription of the eight segments might be due at least in part to feedback control of transcription by the respective protein products.

4. DI RNA

Influenza viruses often contain subgenomic RNA species in addition to the standard eight RNA segments. In 1978, a class of influenza viruses satisfying all the criteria for DI particles (for reviews see References 377 and 387) was identified.[388] Both the multiplicity of infection and the intracellular conditions of host cells affect the generation of DI particles. DI viruses contain one or more novel RNA segments, which become more pronounced on each serially undiluted passage.[388] These RNAs are virus specific, negative stranded, and predominantly of P gene origin.[389,390] Although subgenomic RNA species can be formed randomly for all RNA segments, those of P gene origin might possess some replicative advantage over other subgenomic RNA species. In contrast with DI particles of other virus families, influenza viral DI particles cannot be completely separated from nondefective standard viruses by biochemical procedures and therefore it is hard to determine the RNA segment composition of individual DI particles.

Recently, the complete nucleotide sequences of a number of DI RNAs have been determined. The majority of DI RNAs arise through simple internal deletion, retaining their

original 3′ and 5′ terminal regions, and mainly from P genes.[391-393] In addition, double deletions,[394] complex types (rearrangements within the same progenitor segments)[395,396] have been identified. The results ruled out the splicing mechanism and the posttranscriptional processing of nascent RNA for the generation of DI RNAs. Instead, it was suggested that the RNA polymerase skips a portion of template RNAs during replication. Two models have been proposed: the "jumping polymerase" model, which involves detachment and reattachment of the RNA polymerase to the templates, and the "rolling polymerase" model, which involves looping out of the templates.[387]

B. RNA Polymerases of Bunyaviruses and Arenaviruses

1. RNA Polymerase Genes of Bunyaviruses

The Bunyaviridae is the largest family of RNA viruses including more than 200 serotypes, which are classified into four genera. Bunyaviruses are transmitted to vertebrates by arthropods and pathogenic for human and domestic and wild animals. Bunyaviruses contain a segmented negative-strand RNA genome, consisting of three segments, L, M, and S (for reviews see References 397 and 398). Reassortment of RNA segments has been identified only between closely related viruses. As noted for rhabdoviruses, paramyxoviruses, and myxoviruses, the terminal 3′ nucleotides of the three RNA segments show sequence homology as to 11 to 13 nucleotides,[399] and both terminal sequences are complementary to each other[400-405] and are responsible for the formation of panhandle structures. The lipid envelope contains two external glycoproteins, G1 and G2, which are coded for by the M segment.[406] The three RNA segments are associated with a nucleoprotein, N, which is encoded by the S segment.[407] An RNP-associated large (L) protein is believed to be a component of viral RNA polymerase and a product of the RNA L segment. In contrast to other negative-strand RNA viruses, however, the bunyaviruses lack an internal matrix (M) protein.

In addition to these structural proteins, an NS protein, NS_S, was found to be coded for by the RNA S segment.[408] The results of sequence analyses of S segments from several bunyaviruses indicated that two open reading frames, a larger one for the N protein and a smaller one for the NS_S protein, are overlapped under different reading frames.[401-405] The overlapping gene organization is unique to RNA viruses and one of the economical ways to increase information content. Unlike the cases of the influenza virus M and NS segments, the two proteins seem to be generated through differential utilization of two adjacent initiation codons on the same mRNA molecules. The M segment also codes for another NS protein, NS_M, in addition to the two glycoproteins.[409]

As expected from the negative polarity of genomic RNAs, a viral-associated RNA polymerase was identified for Lumbo virus.[410] Further studies on the structures and functions of bunyaviral RNA polymerases have not been performed. Some bunyaviral mRNA species have heterogenous nonviral sequences at their 5′ termini,[411] suggesting that the RNA polymerases, like influenza viral RNA polymerase, initiate transcription using some cellular RNAs as primers.

Sequence has also been determined for the S segment of Punta Toro virus of phlebovirus genus.[412] The N protein is coded for by the 5′-proximal half of viral complementary sequence (plus-strand transcript); however, a different protein, presumably an NS (NS_S) protein, is encoded by the 5′-proximal half of viral RNA itself. Bishop and colleagues proposed that this type of RNA be designated as "ambisense RNA", i.e., both positive and negative polarity on a single and the same RNA molecule. As described below, a similar structure is known for the genomic RNAs of arenaviruses.

2. RNA Polymerase Genes of Arenaviruses

Arenaviruses are unique in that host ribosomes and small cellular RNA species are incorporated into mature virions.[413,414] The viruses contain three subclasses of RNA: viral-

specific large (L) and small (S) RNAs, host cell ribosomal 28S and 18S RNAs, and ribosome-associated small-molecular-weight RNAs. Arenaviruses have been classified as enveloped segmented negative-strand RNA viruses, the main criteria being the absence of polyadenylated sequences at the RNA 3′ termini, the absence of capped and methylated structures at the 5′-termini, and the absence of messenger activity in an in vitro translation system. Furthermore, polysomal RNA from virus-infected cells is complementary to viral RNAs and can be translated in vitro. In agreement with this interpretation, it was reported that an RNA polymerase activity was present in the purified Pichinde virus of the Arenaviridae.[415,416]

These viruses contain three major structural proteins: two surface glycoproteins, G1 and G2, and one RNA-associated protein, N. These three viral proteins are coded for by RNA S segment, whereas L segment is believed to encode the viral RNA polymerase.[417] The results of sequence analyses have confirmed that a subgenomic viral N mRNA is complementary to the 3′ half of the S RNA, but that a subgenomic glycoprotein precursor (GPC) mRNA is identical to the 5′ half of the RNA.[418] As described above, the term "ambisence RNA" is proposed to describe this novel coding strategy. Although both strands of DNA often contain coding frames, the presence of similar structure in RNA genomes have been identified only for phleboviruses of the Bunyaviridae and arenaviruses. This allows two subgenomic mRNA species to be regulated independently from each other. For example, the GPC mRNA cannot be produced until viral RNA replication or the synthesis of viral RNA (thus the template for GPC mRNA) has commenced. Arenaviruses often cause persistent infections, presumably because viral glycoproteins cannot be synthesized, although the N protein is abundant.

VI. RNA-DEPENDENT RNA POLYMERASES OF DOUBLE-STRAND RNA VIRUSES

Viruses carrying double-strand RNA genomes were characterized as "group 3" viruses[6] and are currently classified as a single family, the Reoviridae. Three genera, i.e., orthoreoviruses, orbiviruses, and rotaviruses, infect animals, whereas the remaining three genera infect insects and plants. Reoviruses lack an envelope and are resistant to lipid solvents, but instead have an outer capsid composed of hexagonal and pentagonal protein subunits. Orbiviruses and rotaviruses are surrounded by a diffuse protein layer.

The double-stranded RNA genomes of all these genera are divided into more than ten segments (for reviews see References 419 to 421). Orthoreoviruses and orbiviruses have 10 segments, and rotaviruses have 11 segments. The RNA segments of mammalian reoviruses can be separated into three size classes, L, M, and S, on sucrose density gradient centrifugation, which can be further separated on gel electrophoresis into 3, 3, and 4 segments, respectively. The size of RNA segments ranges from 1.2 to 4.5 kbp.

A. RNA Polymerases of Reoviruses

Nonionic detergent-treated virions are inactive in RNA synthesis, but an RNA-synthesizing activity was detected in virions after heat treatment[424] or partial protease digestion.[425] Without these treatments, however, virions produce short oligonucleotides,[426] but the elongation of nascent short chains is prevented. Either heat shock or proteolytic cleavage of viral shell might be effective in loosening RNA polymerase-viral RNA complexes. The permeabilized virions continue to synthesize RNA, which protrudes from the virions, the final yield being in excess over the template RNA. The RNA polymerase activity disappears when virions are disrupted; the isolated inner core containing all the RNA segments is inactive, suggesting that the RNA polymerase with a complex structure is located inside the core shell and is disassembled after removal of the outer shell. Enzymatically active cores contain five or six viral proteins, λ1, λ2, λ3, μ2, σ2 (and μ2).[419] Genetic studies suggest that λ3 is the RNA

polymerase protein,[427] but biochemical assignment of the RNA polymerase proteins has not been achieved yet.

Transcripts contain RNA that is hybridizable to all the RNA segments, indicating that at least one molecule of the RNA polymerase is associated with each genome segment. The transcription is asymmetric, i.e., only plus-strand RNA is synthesized, and conservative, i.e., the newly synthesized RNA is released without dissociation of the template duplex. The synthesis of progeny double-strand RNA occurs through transcription of the newly synthesized plus-strand RNA as the template. This conservative mechanism of double-strand RNA replication is in sharp contrast with the semiconservative mechanism of DNA replication.

In addition to RNA polymerase, virions contain nucleoside triphosphate phosphohydrolyase[428,429] and a capping enzyme system comprising RNA-5′ terminal guanylyltransferase, guanine(7-) methyltransferase, and ribose(2′-) methyltransferase.[430,431] All these enzymes are involved in the generation of a cap structure at the 5′ ends of transcripts. The capping activity was also found associated with insect cytoplasmic polyhedrosis virus (CPV).[432] The capping reaction catalyzed by the reovirus-associated enzymes is essentially identical to that by vaccinia virus-associated enzymes, as discussed in Section II. The catalytic unit of guanylyltransferase, an enzyme involved in the cap formation, was identified as λ2, encoded by RNA segment L2, by analysis of a covalent enzyme-guanylate intermediate.[433]

VII. CONCLUDING REMARKS

The recent progress of gene cloning and nucleic acid sequencing techniques has led to striking advances in our understanding of viral RNA polymerases.

1. Vaccinia virus-associated RNA polymerase holoenzyme is composed of six or seven polypeptide subunits. The largest subunit contains structural domains that are highly conserved in the largest subunits of cellular RNA polymerases from animals, insects, yeast, and bacteria. The holoenzyme recognizes virus-specific promoters and faithfully transcribes cloned vaccinia viral genes.
2. The RNA polymerase core enzyme from poliovirus-infected cells consists of the viral NS protein 3D, which is encoded by the 3′-proximal gene of polioviral RNA. A host factor, presently identified as terminal uridylyltransferase, donates primers for the initiation of minus-strand RNA synthesis, whereas a viral protein VPg acts as a protein primer for the initiation of plus-strand RNA synthesis.
3. Togaviruses contain NS protein genes in the 5′-proximal region of single- and positive-strand genome RNA but flaviruses in the 3′-proximal region. The sequences conserved in RNA polymerase genes, including the canonical Gly-ASP-ASP sequence, are present in the 3′-proximal region within the respective NS protein gene clusters.
4. The RNA polymerase holoenzyme from VSV is composed of two viral proteins, L and P (previously designated as NS). The catalytic sites for RNA synthesis, capping and poly(A) synthesis are located on the L protein, while the P protein is needed for elongation of RNA chains. Viral protein N regulates read-through transcription at intercistronic boundaries and the M protein prevents the expression of RNA polymerase activity.
5. The structures and functions of RNA polymerases from paramyxoviruses, another nonsegmented negative-strand RNA virus family, are similar to those of VSV RNA polymerase. In agreement with the growth characteristics of this group of viruses, the RNA polymerase activity is influenced strikingly through interaction with cellular cytoskeletal proteins.
6. Influenza viral RNA polymerase is tightly associated with each of the eight minus-strand RNA segments. The enzyme is composed of three P proteins, PB1, PB2, and

PA, and catalyzes multiple reactions, including the capped RNA cleavage, primer-dependent RNA synthesis, and poly(A) synthesis, leading to the formation of viral mRNAs. The enzyme seems to be functionally modulated to an RNA replicase by a yet unidentified mechanism.

7. Both arenaviruses and bunyaviruses contain segmented single-strand RNAs as the genomes, some of which are ambisence RNAs, i.e., mosaics containing both plus- and minus-strand on the same RNA strands. Virions of these viruses contain virion-associated RNA polymerases as well.
8. RNA polymerases of reoviruses are inner-surface components of the virion outer shells and are inactivated upon disruption of virions. The RNA polymerase transcribes double-strand RNA segments in a conservative manner and, in addition, modifies nascent transcripts with a cap structure and a poly(A) tail.

Detailed understanding of the structures and functions of viral RNA polymerases await more direct approaches, such as the purification and characterization of RNA polymerases and the genetic and biochemical dissection of polymerase proteins, including site-directed mutagenesis and expression of cloned polymerase genes.

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