MICROBIOLOGICAL REVIEWS, Mar. 1987, p. 66–87
0146-0749/87/010066-22$02.00/0

Vol. 51, No. 1

Transcription and Replication of Rhabdoviruses

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INTRODUCTION

Rhabdoviruses belong to the special class of viruses whose linear single-strand genome ribonucleic acid (RNA) is of negative polarity; i.e. their genome RNA is noninfectious and complementary to functional, virus-specific, positive-sense messenger RNAs (mRNAs) (233). These characteristically bullet-shaped viruses (122, 146) are ubiquitous in nature and have a uniquely broad and diverse host range including vertebrates, invertebrates, and plants. Two notable and often studied rhabdoviruses are the animal pathogen vesicular stomatitis virus (VSV) and the human pathogen rabies virus, although a number of laboratories have initiated the study of fish and plant rhabdoviruses. VSV, however, still remains the prototype of rhabdoviruses and is widely used in the laboratory for biochemical studies, primarily due to its availability in a highly purified form and in high yield from infected cells. Consequently, the discussions within this review on the biology and gene expression of rhabdoviruses are intimately linked to the information generated through extensive study on VSV over the past 15 years. In this review I shall discuss the current state of knowledge about rhabdoviruses with regard to the in vitro studies of transcription and replication of VSV as the prototype rhabdovirus. However, references to results obtained with other rhabdoviruses of both animal and plant origin will be made whenever appropriate. Interested readers are referred to earlier and more recent reviews which have dealt with either rhabdoviruses in general or some specific aspects of transcription and replication of VSV (6, 11, 13, 14, 27, 71, 178, 233, 237). Recently, the biology, structure, and replication of plant rhabdoviruses have been reviewed (A. O. Jackson, R. I. B. Francki, and D. Zuidema, *in* H. Fraenkel-Conrat and R. R. Wagner, ed., *The Viruses*, in press).

One of the unique features of the negative-strand genome RNA of a rhabdovirus is that it serves as the template for both transcription (mRNA synthesis) and replication (genome RNA synthesis). Moreover, due to the packaging of a novel, virus-specified RNA polymerase within rhabdovirions (10), these viruses serve as one of the model systems to study transcription and replication and their regulation in vitro. The linear, single-strand genome RNA of VSV (molecular mass, 4×10^6 daltons [Da]) is tightly associated with monomers of the nucleocapsid protein N (49 kDa) which are closely packed along its length. Together with two minor proteins, L (241 kDa) and phosphoprotein NS (29 kDa), the genome N-RNA complex constitutes the transcribing ribonucleoprotein particle (RNP). The purified RNP synthesizes virus-specific mRNAs in vitro (10, 25, 141), and in vivo this RNA-synthetic process is responsible for successful initiation of infection through primary transcription and subsequent secondary transcription, which are qualitatively identical and lead to replication of the genome RNA (178). Within the virion the RNP is coiled to form a tight helix and is associated with the matrix protein, M (25 kDa), which is beneath the lipid bilayer acquired from the host. The glycoprotein, G (69 kDa), projects outside the lipid bilayer and is the antigenic determinant of the virus. Recent studies with dark-field scanning transmission electron microscopy have revealed that the genome RNA of VSV is associated with 1,258, 466, and 50 copies of the N, NS, and L proteins, respectively, whereas the amounts per virion of the G and M proteins are 1,205 and 1,826 molecules, respectively (222). These numbers are different from values previously determined by biochemical methods (26): molecules per virion—

N = 2,300; NS = 230; L = 60; G = 2,100; and M = 4,700. The polypeptide compositions of fish rhabdoviruses and rabies virus are very similar to that of VSV, except that for rabies virus both M1 (the equivalent of NS) and the nucleocapsid protein (N) appear to be phosphorylated (46, 47, 59, 67, 132, 225, 229, 245). The polypeptides of plant rhabdoviruses (58, 247), although considerably different in both size and polyacrylamide gel electrophoresis patterns, are similar to animal rhabdoviruses and follow the nomenclature proposed for the latter (Jackson et al., in press).

An important feature of VSV is that the individual viral proteins and the N-RNA complex can be obtained in a highly purified form. Emerson and Wagner (72) were the first to successfully separate and isolate the polypeptide components of purified RNP into the L and NS proteins and the N-RNA complex. Addition of these individual components to each other reconstituted the RNA-synthetic capability of the RNP. Furthermore, it was shown that both the L and the NS proteins are required for RNA synthesis in vitro, and an equimolar proportion of each of the proteins was reported to be needed for optimal in vitro transcription (73, 150). These observations led to further studies to understand the role of individual proteins in the RNA-synthetic process and the mechanism of RNA transcription in vitro. Recently, using recombinant deoxyribonucleic acid (DNA) technology, each of the mRNAs coding for the viral proteins has been molecularly cloned, and through the work of several laboratories, the nucleotide sequences have been determined (53, 80, 137, 138, 184, 185, 188, 194, 198). In fact, the complete nucleotide sequences of the genome RNA of the Indiana serotype of VSV and of half of the genome RNA of the New Jersey serotype of VSV are now known (18, 55, 81, 84). More than half of the rabies virus genome RNA sequence has also been determined recently (N. Tordo and O. Poch, personal communication).

TRANSCRIPTION OF RHABDOVIRUSES

In Vitro Transcription System

Baltimore et al. (10) made the original discovery that purified VSV contains a virion-associated RNA-dependent RNA polymerase that transcribes RNA in vitro complementary to the virion RNA. Subsequently, it was shown that detergent-disrupted purified virions (25, 141), purified RNP (1, 212), or RNP isolated from infected cells (79, 225) are all capable of synthesizing RNA in vitro in the presence of the four ribonucleoside triphosphates. For optimal transcription, the requirement for adenosine triphosphate (ATP) is considerably higher (K_m = 0.5 mM) than that for the other three ribonucleoside triphosphates (K_m = 0.05 mM) (218). This higher requirement for ATP is probably due to its role in the initiation of RNA synthesis or its utilization in phosphorylation of NS protein (see below). Curiously, in the VSV transcription reaction deoxyguanosine triphosphate (dGTP) can substitute for guanosine ribonucleoside triphosphate (GTP), giving a similar yield of RNA synthesis in vitro (199) and thus rendering the product RNA resistant to ribonuclease (RNase) T_1 digestion. However, the reasons for the specific utilization of dGTP by the virion-associated RNA polymerase remain unknown. Similarly, guanosine diphosphate (GDP) can substitute for GTP in the transcription reaction mixture (219). In the latter case, it was shown that purified virions contain a nucleoside diphosphokinase which converts GDP to GTP. Whether this enzyme is virus coded or a cellular contaminant is not clear.

An important parameter of the in vitro transcription system is the requirement for NaCl. Using detergent-disrupted virions and lowering the optimal salt concentration (0.1 M to 0.02 M) results in a drastic reduction of RNA synthesis in vitro. In contrast, RNA synthesis by purified RNP free of G and M proteins was found to be insensitive to a reduction of salt concentration (65, 172). It was later shown that the M protein, a major constituent of the virion, interacts with the RNP under low salt concentration, resulting in the cessation of RNA synthesis (see below). This result indicates that the matrix protein modulates RNA synthesis in vitro and probably plays a regulatory role in infected cells.

Since highly purified RNP transcribes mRNA efficiently in vitro, it is generally assumed that VSV transcription is not dependent on any host cell component. However, stimulation of RNA synthesis in vitro by the addition of host cell extract has been reported (8, 187). Recently, β-tubulin has been implicated as a positive transcription factor for VSV since in vitro mRNA synthesis is completely abolished by the addition of β-tubulin antibody (142a). In addition, the L protein has been shown to be associated with β-tubulin when the latter is immunoprecipitated from infected cells. However, the presence of β-tubulin in purified virions has not been rigorously demonstrated. Further studies are needed to elucidate the precise role of β-tubulin in VSV transcription.

The RNA-synthetic activity of purified RNP of VSV has also been demonstrated in various serotypes in addition to the widely used Indiana serotype (43, 54, 78). Similar activity has also been shown for rabies virus (76, 115), although this activity is significantly lower than that of VSV. RNA-dependent RNA polymerase activities have been demonstrated in three salmonid rhabdoviruses: infectious hematopoietic necrosis virus, virus of hemorrhagic septicemia, and spring viremia of carp virus (91, 136, 189, 190). The optimum temperatures for in vitro transcription for these viruses range from 15 to 20°C in contrast to 30°C for VSV. The best-studied plant rhabdovirus, Sonchus yellow net virus, also contains a virion-associated RNA polymerase which synthesizes RNA in vitro at 25°C, as well as in vivo (179; P. Flore and D. Peters, personal communication). Transcriptase activities have been shown in broccoli necrotic yellows virus and lettuce necrotic yellows virus (77, 226, 227), although the in vitro RNA products of these viruses have not been adequately characterized. Further studies on such plant rhabdoviruses would certainly help our understanding of the mechanism of gene expression of these plant pathogens and of their similarities or dissimilarities to the animal rhabdoviruses.

mRNA Synthesis and Post-Transcriptional Modifications

Five distinct mRNA species are synthesized in vitro by detergent-disrupted VSV virions or purified RNP, and each can be identified by in vitro translation as coding for one of the five structural proteins L, NS, M, G, and N (32–34). Similarly, viral mRNAs from the infected cells are separable into five distinct species which are translated in vitro to produce all virus-specified proteins (34, 121, 144, 186). Thus, the virion-associated RNA polymerase faithfully transcribes the genome RNA both in vitro and in vivo.

The in vitro synthesized mRNA species contain the hallmarks of typical eucaryotic mRNAs, i.e., a 5′-terminal cap structure (GpppA) (4, 95) and a 3′-polyadenylic acid [poly(A)] tract (16, 68, 79, 232). In the presence in vitro of the methyl donor *S*-adenosyl-L-methionine (AdoMet), spe-

cific methylation of the 5'-cap structure occurs with the formation of the dimethylated cap 5'-$^{7}_{m}$GpppA^{m} . . . (3). In contrast, the mRNAs isolated from infected cells contain, in addition to methylated G, a doubly methylated penultimate A (at the 2'-O-ribose and the N^6A-adenosine positions) and variable degrees of 2'-O methylation at the second A residue (143, 183). In addition, uncapped full-length transcripts containing 5'-triphosphate A and G have been found in low levels in vitro (95, 225) and in infected cells (183). The unique feature of the cap structure of the VSV mRNAs is that the α and β phosphates of the blocking GTP and the α phosphate of the penultimate A are incorporated into the 5'-5'-triphosphate moiety G(5')αβ|α(5')AA (3, 4). This feature is quite different from those of other well-established viral systems, such as reovirus and vaccina virus (12), in which the α phosphate of the blocking GTP and both α and β phosphates of the penultimate base are incorporated into the triphosphate bridge. This observation initially lent credence to the idea that the biosynthesis of VSV mRNAs in vitro might be mediated by a cleavage process (see below). However, in a related fish rhabdovirus, spring viremia of carp virus, Gupta and Roy (89) demonstrated that in vitro transcripts contain the α phosphate of GTP when the GTP analog 5'-guanylylimidodi-phosphate (GMPPNP) was used as a ribonucleoside triphosphate precursor, whereas in the presence of GTP the expected α and β phosphates of GTP were incorporated into the capped structure. These results indicate that the virus may use alternate capping pathways depending on the availability of the hydrolyzable phosphates in the GTP used as substrate.

Two distinct methyltransferase activities have been detected in vitro in purified VSV virions (216). At a low concentration of AdoMet, the penultimate A residue is methylated at the 2'-O-ribose position, whereas at a higher concentration the blocking G is methylated. In the presence of *S*-adenosyl-L-homocysteine, the methylation of guanosine is inhibited prior to ribose methylation of A. This pathway of methylation in the VSV system is again different from those of the well-studied reovirus or vaccinia virus (for a review, see reference 12), in which methylation of the blocking guanosine precedes ribose methylation of the penultimate base. However, by using cycloleucine, an inhibitor of methylation in vivo, it has been shown that 2'-O methylation is inhibited prior to 7-methylation of the blocking G (35, 142). These results may indicate that each of the two methyltransferases in VSV demonstrates a different K_m for AdoMet in vitro and in vivo. Alternatively, there may be a high enough concentration of host methylase in the cytoplasm to ensure that the blocking G is methylated first. In the fish rhabdovirus spring viremia of carp, both AdoMet and *S*-adenosyl-L-homocysteine are found to stimulate in vitro RNA synthesis (90). In addition, purified virions appear to contain some form of endogenous AdoMet, the exact chemical nature of which remains to be elucidated (90).

The in vitro synthesized mRNAs contain approximately 200 A residues at their 3' end, indicating that post-transcriptional polyadenylation is carried out by one or more virus-specific proteins (16, 68, 79, 232). Polyadenylation is probably carried out by a "slippage" mechanism in which the polymerase repeatedly transcribes a stretch of U sequences located at the end of each gene (see below). Curiously, *S*-adenosyl-L-homocysteine, an inhibitor of methylation, greatly increases the length of the poly(A) tails on VSV mRNAs made in vitro (187). Although the role of *S*-adenosyl-L-homocysteine has not been elucidated, the results seem to indicate that methylation and polyadenylation are interdependent and both may have roles in the transcription process.

Leader RNA

In addition to the five distinct VSV mRNAs synthesized in vitro, an additional unique RNA was discovered in the transcription mixture (51). This species, termed leader RNA, is 47 bases long and represents an exact complement of the 3' terminus of the genome RNA (52). In contrast to the mRNA species, the leader RNA has a polyphosphorylated 5' end and does not contain poly(A) at its 3' end (53). Subsequently, it was found that a VSV DI particle (55a) and several unsegmented negative-strand RNA viruses, including rabies virus (125), contain a domain of variable size at the precise 3' ends of their genome RNAs which are transcribed in vitro and in vivo as leader RNAs. Recently, a plant rhabdovirus, Sonchus yellow net virus, has also been shown to contain such a leader template, approximately 140 bases long, in the genome RNA (248). The unique size and the transcriptional map position of the leader RNA on the genome RNA indicate that it must have a specific function in transcription or replication (see below).

In Vitro Transcription Process

The first indication that the mode of transcription of VSV genome RNA in vitro is complex came from the following observations. (i) All mRNA species are initiated with an identical 5'-terminal sequence, G(5')ppp(5')AACCAG (182), whereas the 3'-terminal sequence of the genome RNA is 3' UGC, which is not complementary to any of the mRNAs (17). (ii) The synthesis of mRNA species, both in vitro and in vivo, is nonequimolar; i.e., the synthesis of N mRNA is in the highest molar amount, followed by NS, M, G, and L, in that order (180, 181, 231). (iii) The unique 47-base-long leader RNA is the first product to be synthesized in vitro (52). From these observations it became apparent that the initiation of RNA synthesis occurs at the 3' end of the genome RNA with the synthesis of the leader RNA, and the mRNA species are synthesized subsequently. The 5' capping and 3' polyadenylation are probably mediated by viral polypeptide components of the RNA polymerase complex.

UV Transcriptional Mapping and Gene Order

To determine the spatial relationship between the individual viral genes and their promoter sites, the technique of ultraviolet (UV) transcriptional mapping was used (1, 7). Measurement of the UV sensitivity of the expression of individual VSV genes in vitro clearly demonstrated that the measured target sizes are very close to the cumulative physical sizes summed in order of increasing UV sensitivity: N < N + NS < N + NS + M < N + NS + M + G < N + NS + M + G + L. The simplest interpretation of this result is that the order of UV sensitivity reflects the physical order of the genes within a single transcriptional unit. Thus, the five genes are transcribed sequentially in the order N-NS-M-G-L, and the initiation site for RNA synthesis might be at the 3' terminus of the viral RNA. Since a similar decreasing order in the molar yields of individual mRNAs is seen in vitro, the transcription of the genes must be discontinuous. Transcriptional mapping studies of the leader RNA showed that its UV target size is equivalent to its physical size (0.02 × 10^6Da), which suggests that its location is very close to a promoter site and that it is transcribed before the five

structural genes. In fact, the leader RNA is present in the highest molar amount among the reaction products (52). However, due to its very small target size, it makes no detectable contribution to those of the distal genes; consequently, it could not be definitively assigned to the same transcriptional unit. Nevertheless, the simplest interpretation of the above results is that the transcription initiation occurs at the 3′ end of the genome RNA with the synthesis of the leader RNA followed by the sequential synthesis of the five mRNAs. Thus, the gene order of the VSV genome RNA is 3′ leader RNA-N-NS-M-G-L 5′ (1, 7), which was subsequently confirmed by Reddy et al. (177) and by electron microscopic analysis (96). By similar UV transcriptional mapping studies, the gene order of rabies virus was established as (3′) leader RNA-N-M1-M2-G-L 5′ (75). It is of interest to note that a fish rhabdovirus, spring viremia of carp virus, appears to have the matrix protein gene (M) next to N protein gene rather than the phosphoprotein gene (NS) as found in other rhabdoviruses (P. Roy, personal communication). The precise implication of these results must await further studies.

Consistent with the presence of five genes in VSV, genetic analysis also revealed that the temperature-sensitive (*ts*) mutants fall into five complementation groups (74, 174). However, similar mutants obtained from the New Jersey serotype of VSV have been shown to constitute six genetically well-defined complementation groups (176), four of which have an RNA-negative phenotype; these mutants do not synthesize RNA at the nonpermissive temperature. This suggests either that a fourth viral protein participates in RNA synthesis or that two groups of mutants are affected in different RNA-synthetic functions residing in the same protein. By using reconstitution experiments (153) and a hybrid infectivity assay (23), it was resolved that two complementing mutants, B1 and F1, both have *ts* lesions in the L protein. Thus, it appears that the L protein has at least two functions in transcription, and these functions can be separately mutated and can intragenically complement each other.

On the other hand, other rhabdoviruses have been shown to contain six genes. By molecular cloning, six distinct mRNA species were characterized in infectious hematopoietic necrosis virus, a salmonid rhabdovirus (124). In addition, R-loop mapping with complementary DNA (cDNA) plasmids determined that the gene order on the infectious hematopoietic necrosis virus genome is 3′ N-M1-M2-G-NV-L 5′ (123). The M1 and M2 proteins are the analogs of the phosphoprotein (NS) and the matrix protein (M) of VSV, respectively, whereas NV is a nonvirion protein. The NV protein has a mass of approximately 12 kDa and is encoded by an mRNA of 570 bases. Thus, the fish rhabdoviruses may contain an additional gene, the function of which is not known at present. By direct sequencing of the rabies virus genome, an additional genetic element was found between the G and L genes (Tordo and Poch, personal communication). Recently, in a strain of the New Jersey serotype of VSV, the sequence at the start of the L gene was found to be different from the rest of the genes (D. S. Gill, unpublished observation), indicating that either there is a different start signal for the L gene or additional genetic material is present at that intragenic region. Further studies are clearly needed to gain insight into the structure and function of these putative rhabdoviral sixth genes.

Discontinuous RNA Synthesis In Vitro

Despite the fact that transcription is sequential, the five VSV mRNAs are, as described above, present in nonequimolar ratios both in vitro and in vivo (180, 231). Since, in vivo, the rates of degradation of each of the VSV mRNAs are the same (163), the differences in the abundance of viral mRNAs must be due to differential rates of synthesis. Direct kinetic analyses of transcription in vitro show sequential appearance of mRNAs in the order leader RNA, N, NS, M, G, and L (39, 114, 221), with significant pauses (attenuation) between the transcription of contiguous genes (113). Transcription appears to decrease by 29 to 33% across the N-NS, NS-M, and M-G gene junctions, resulting in a cumulative effect on gene expression. It has been suggested that pauses may occur during polyadenylation or may be due to some other process, such as initiation or capping, which is slow relative to transcription (113). However, it should be noted that the leader RNA is not polyadenylated, and thus pausing between transcription of the leader template and the N gene cannot be due to polyadenylation. Although the mechanism of this attenuation phenomenon is not clearly understood, the observed transcription attenuation in the VSV system may be the principal means by which the virus regulates mRNA and protein syntheses during its replicative cycle.

Models for RNA Synthesis In Vitro

The unique polar effect on transcription in vitro described above suggested that the virion-associated polymerase most likely initiates at a single site at the 3′ end of the viral genome. Coupled with the observation that the capping reaction involves an interaction between GDP and a 5′-monophosphate-terminated mRNA (4), the simplest interpretation was that the mRNAs are formed in vitro by cleavage of the growing nascent chain initiated at the 3′ end of the genome RNA (9, 13) (Fig. 1, cleavage model). Cleavage was proposed to occur at specific sites on the growing chain followed by capping of the 5′ ends of the mRNA chains and polyadenylation at the released 3′ ends. This model lacked support mainly due to insufficient data demonstrating cleavage reactions or the existence of large uncleaved precursor RNAs in vitro. Moreover, the decreasing synthesis of RNA species in vitro in the order, leader RNA > N > NS > M > G > L (2) clearly indicated that some additional steps besides processing are involved in the biosynthesis of individual mRNAs. However, several observations still remain compatible with the cleavage model. Low but consistent levels of polycistronic, or readthrough, RNAs are found in VSV-infected cells (135). Interestingly, the frequency of readthrough between the NS and M genes has been found to be higher than that at the other junctions. Even the readthrough products from the L gene into the extracistronic region up to the 5′ terminus of the VSV genome RNA have been detected in vivo (98, 196). Whether these readthrough products are unprocessed mRNA precursors or are produced as a result of abortive replication attempts by the transcriptase is unknown. By using 5′-adenylylimidodiphosphate (AMPPNHP), γ-thio-ATP, or inosine triphosphate in the in vitro transcription reaction, it is possible to detect RNA species containing leader RNA covalently linked to the N gene and beyond (41, 44, 99, 217, 220). In fact, complete readthrough of the full-length positive polarity strand has been observed in vitro (41, 220). In addition, low levels of polycistronic RNAs containing intervening poly(A) stretches have also been found among in vitro RNA products (96). Failure to process readthrough RNA transcripts in vitro supports the idea that these RNA molecules are probably generated by a replication attempt by the RNA polymerase.

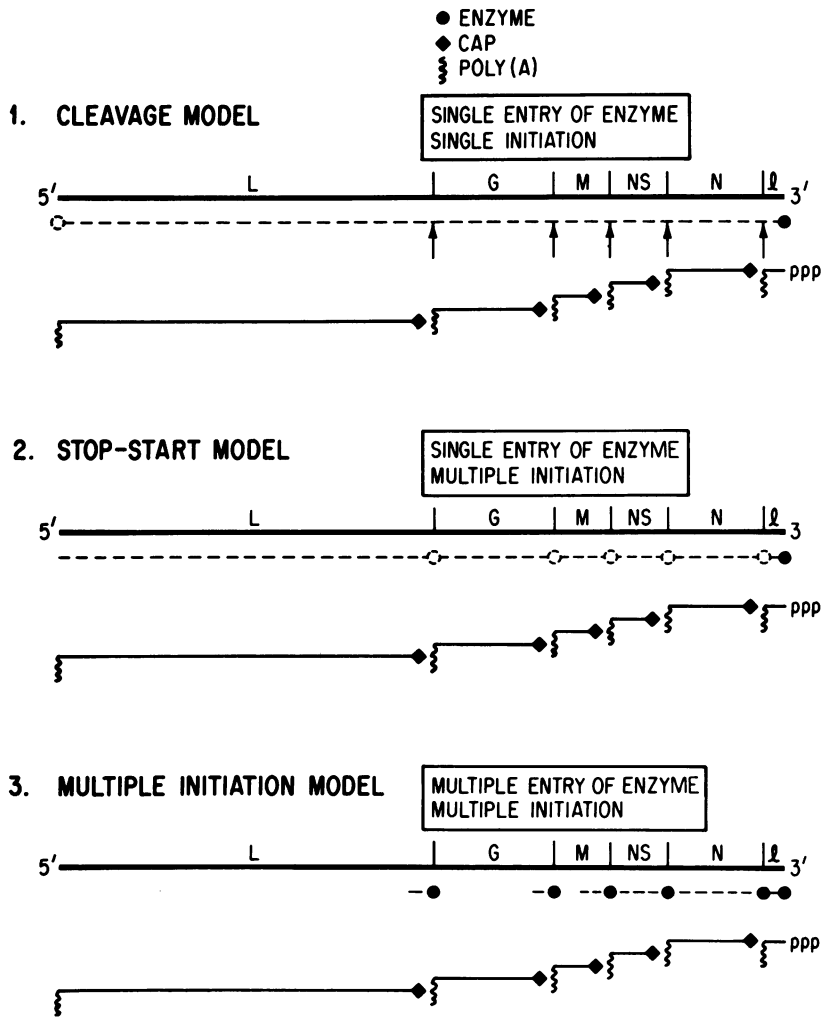

FIG. 1. Models for VSV RNA synthesis. Arrows in the cleavage model indicate the putative cleavage sites.

The second model, termed the stop-start model (Fig. 1), proposed that the virion-associated RNA polymerase initiates RNA synthesis at the 3′ end of the genome RNA and synthesizes the leader RNA first. The same enzyme then reinitiates an RNA chain on the N gene and continues its synthesis until it terminates chains at the 3′ end and adds poly(A) by slippage on the U residues present at the intragenic junction. The same enzyme continues synthesis of NS mRNA by reinitiation. The process continues until the enzyme reaches the end of the L gene. During its journey from the 3′ to the 5′ end of the genome, the enzyme may fall off the template at any point or at junction points but may bind only at the 3′ end of the genome RNA to initiate RNA synthesis. This process will generate a concentration gradient of the RNA species in the order, leader RNA > N > NS > M > G > L. The crux of this model is that there is an obligatory requirement for the virion-associated RNA polymerase to enter at a single site at the 3′ end of the genome RNA throughout the transcription process. An observation which inspired Emerson (70) to postulate this model is that a defective interfering (DI) particle, HR-LT2, which contains a leader gene located approximately 70 nucleotides from the 3′ end of the genome RNA fails to transcribe the leader RNA in vitro (116, 177). Thus, the location of the leader gene at the 3′ end appears to be essential for binding of the polymerase to initiate transcription. Experimental evidence for the stop-start model has been provided by Emerson (70), who demonstrated that in an incomplete reaction condition containing ATP and cytosine triphosphate, the reconstituted RNP complex synthesized only dinucleotide pppAC representing the 5′ dinucleotide of the leader RNA. The oligonucleotide pppAACA, representing the 5′-terminal sequence of the mRNAs, was synthesized in a similar partial reaction only after RNA synthesis by the reconstituted complex was allowed to proceed in the presence of all four ribonucleoside triphosphates (thus allowing the enzyme to move on the template).

Although this is an attractive and highly plausible model, some published observations seem to be inconsistent with it. For example, the model predicts that initiation on the leader template must precede initiation on the N gene. However, certain in vitro transcription conditions such as low salt (171), the presence of matrix protein (65, 172, 223), or the presence of the inhibitor aurintricarboxylic acid or vanadyl ribonucleoside complex (215) allow preferential synthesis of capped and uncapped oligonucleotides representing the 5′ terminus of the N-mRNA. Moreover, synthesis of pppAC and pppAACA may not necessarily represent the initiating sequences of the leader template and the N gene, respectively. Within the leader template there are domains where

the virion-associated RNA polymerase may initiate and synthesize such di- and tetranucleotides. In fact, in partial reactions (minus uridine triphosphate and GTP), additional oligonucleotides such as ACC and ACAA are synthesized in substantial amounts (223) and probably arise from within the leader template. Finally, although the model explains the polarity of transcription, it does not address the phenomenon of attenuation of RNA synthesis in vitro; a reason for the polymerase to pause at the end of the leader template before initiating the N gene remains unresolved. Nevertheless, the stop-start mechanism of RNA synthesis is an attractive one, and it suggests that both the virus transcriptase and replicase may recognize the same initiation site. Thus, this specificity would not require that the replicase discriminate between the 3′ site and internal polymerase-binding sites.

The multiple (independent) initiation model, on the other hand, proposed that transcription initiation occurs at multiple internal sites in addition to the leader template (Fig. 1, multiple initiation model) (221). RNA synthesis thus begins by multiple initiations at putative promoter sites, but the elongation of each small internally initiated RNA into its corresponding mature message depends on the prior transcription of the 3′-proximal gene. This pausing of initiated transcripts may form the basis of the observed attenuation of RNA synthesis. The secondary structure of the transcribing RNP is proposed to play a role in this cascade mode of transcription. This model basically agrees with the stop-start model in having each mRNA result from a separate initiation event, but it differs in that these initiations are proposed to be independent. The bulk of the evidence for this model comes from the fact that, in addition to the leader RNA, distinct 5′-polyphosphorylated transcripts representing the 5′ termini of the N-mRNA and the NS-mRNA are found in the products of transcription reactions (40, 221). These transcript termini are synthesized very early during transcription and continue to be synthesized with time, whereas the mature mRNAs appear later and sequentially. Multiple internal bindings of the polymerase also have been demonstrated in reconstitution experiments (223). The N-RNA complex was first irradiated for different periods of time and subsequently reconstituted with active soluble transcriptase. Although the extent of RNA synthesis was decreased >80%, the synthesis of the leader RNA and several initiated transcripts representing the N- and NS-mRNA termini remained virtually constant. These results suggest that each of the RNA species is initiated through independent binding of the transcriptase at its putative promoter site. In addition, during reconstitution at low ionic strength, and in the presence of matrix protein (M), synthesis of the leader RNA is abolished, while the synthesis of the small initiated N-mRNA transcripts remains unaffected.

The involvement of the secondary structure of the transcribing RNP was demonstrated by using the photoreactive compound 4-substituted psoralen (151). Photoreaction modifies the genome RNA within the N-RNA complex such that, at a concentration of 10^{-4} M psoralen, the transcription of genome RNA in vitro is inhibited by >90%. By using ^{3}H-labeled psoralen, it was shown that photoreaction occurs at a cytosine residue within the N gene near the 3′ end of the viral genome RNA (214). Binding of psoralen to a specific site on the N gene eliminates not only the formation of the N-mRNA but also the transcription of the other viral genes. The synthesis of the small initiated transcripts, however, continues. The replication of the virus may be tied in with this model. If the attenuation of the leader RNA is somehow relieved, either by interaction with the N protein or phosphorylation of a virion protein, the leader RNA may now be extended beyond its early termination site with the synthesis of the full-length complementary strand (42S positive strand). This would enable the structure of the RNP to be altered such that internal initiations would be compromised.

Like the stop-start model, this model also suffers from some deficiencies. Iverson and Rose (114) failed to detect such small uncapped transcripts in their in vitro reaction conditions and observed sequential appearance of leader RNA followed by the N-mRNA sequences. Piwnica-Worms and Keene (173), on the other hand, detected similar transcripts in vitro but, in addition, found two capped RNA species corresponding to the termini of the N-mRNA and the NS-mRNA. Unlike the 5′-triphosphate-containing transcripts, these capped RNAs appear sequentially and have UV target sizes greater than their respective lengths. These results would indicate that the N- and NS-mRNAs are synthesized concominantly rather than simultaneously. Moreover, the multiple initiation model is based on the assumption that the small RNAs are indeed mRNA precursors. Attempts to chase the initiated transcripts into mature mRNAs thus far have failed (40, 127). This suggests that these RNA transcripts may be abortive products of transcription, although their existence implies the presence in the VSV genome of internal promoter sites. The function of these transcripts, if not for message synthesis, remains a mystery (6). It is noteworthy, in this context, that Talib and Hearst (215) demonstrated that in the presence of aurintricarboxylic acid and vanadyl ribonucleoside complex mRNA synthesis by purified RNP was totally inhibited, but a 68-base capped RNA corresponding to the 5′-terminal transcription product of the N gene continued to be synthesized. The termination of transcription occurred precisely at the 118th base from the 3′ end of the VSV genome. This differential inhibition of leader RNA relative to the N-mRNA proximal sequence by aurintricarboxylic acid and vanadyl ribonucleoside complex suggests that perhaps two types of polymerase activities are involved in VSV transcription: one which is involved in the synthesis of the leader RNA and is more sensitive to these inhibitors, and another which is involved in the initiation of mRNAs and is less sensitive to the same inhibitors. It is significant that Schubert et al. (195) characterized a triphosphate G-start RNA initiating at the 78th base from the 3′ end of the genome RNA and terminating at the identical 118th base. This result also suggests that internal initiations occur during transcription. However, the functions of these seemingly abortive RNA species in the VSV transcription process still remain unclear.

Thus, the precise mechanism by which the VSV mRNAs are synthesized from the genome template remains unresolved. Both models agree that the synthesis of each mRNA results from separate initiation events, but they differ in that these initiations are either independent (multiple entries of enzyme) or sequential (single entry of enzyme). It seems that a hypothesis combining the two models can be envisaged in which multiple entry of one subunit of the polymerase, for example, L, occurs at internal promoter sites followed by a preferential entry of the other subunit, NS, at the 3′ end, which results in initiation of synthesis of the leader RNA. The sequential mRNA synthesis occurs due to an increased (stoichiometric) requirement for the NS protein in order for the L protein to initiate downstream genes. A better understanding of the VSV transcription process will certainly be achieved by studying the exact locations of the polymerase

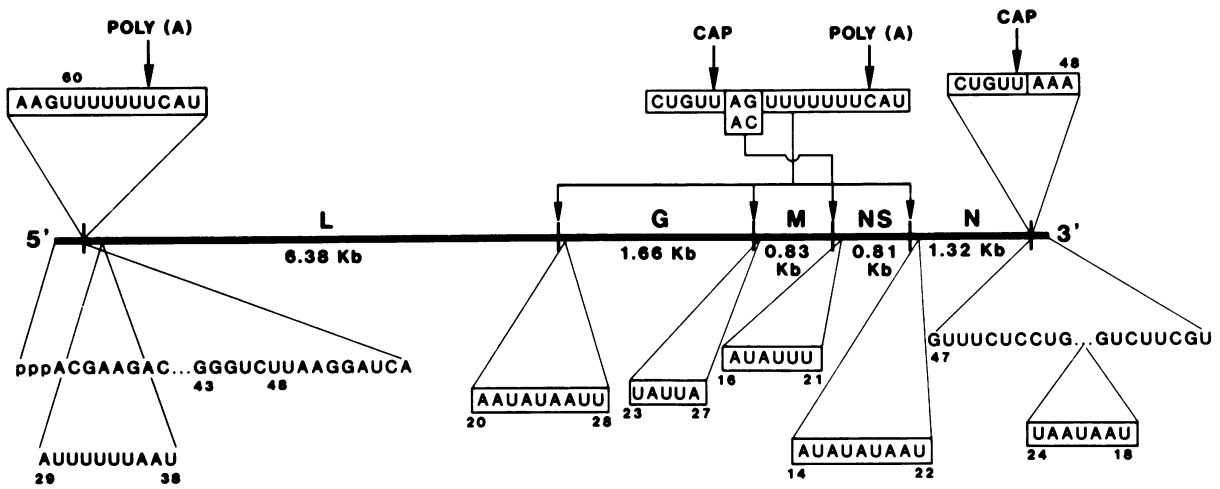

FIG. 2. Physical map of VSV (Indiana) genome. L, G, M, NS, and N correspond to viral structural proteins. The 47 bases at the 3′ end represent the region coding for the leader RNA. The boxed nucleotide sequences (below the genome) are the AU-rich sequences present in the genome RNA. The boxed nucleotide sequences (above the genome) are the intragenic sequences, as indicated. The G is replaced by C in the corresponding sequence between the M and NS genes. kb, kilobases.

molecules on the N-RNA template. Recent electron micrographic techniques (92) indicate that the L and NS proteins are uniformly distributed throughout the VSV RNP, with more than twice as many NS molecules than L molecules present per RNP. Clearly, similar experiments need to be carried out by binding individual proteins to the N-RNA complex during or in the absence of transcription. Whatever the mechanism used by the transcriptase of VSV for generating the RNA transcripts, an important point to note is that there must exist some types of signals indicating the beginning and end of each gene. These signals are only observed when the RNA polymerase is operating in the transcriptive mode. To synthesize a complete 42S positive strand during the first stage of replication, it is essential to suppress the recognition of the internal start and end signals such that the RNA polymerase can assume the replicative mode or, alternatively, the template itself may assume a replicative form (see below).

It should be noted that none of the models address some important observations made regarding the in vitro transcription reaction. (i) The leader RNA is not capped, whereas the mRNAs are (53). Since both L and NS proteins are involved in the transcription process, it is difficult to envisage how the capping activity is compromised during initiation at the 3′ end. (ii) In the partial in vitro transcription reactions, the mRNA start oligomers (AACA, AACAG, etc.) are uncapped (40, 70). This suggests that the capping reaction occurs following initiation of mRNA synthesis. In fact, the shortest capped mRNA fragment isolated from the in vitro product ranges from 20 to 68 bases (40, 173). Thus, the mechanism of how the capping of mRNAs is regulated in vitro remains unresolved. (iii) ATP seems to play an important part in the initiation of RNA synthesis. Little, if any, RNA is synthesized if ATP is replaced with AMPPNHP in the transcription mixture (168, 218). In one report (88), leader RNA and small uncapped N-mRNA transcript termini were synthesized in the presence of AMPPNHP, but no full-length mRNAs were detected. These results indicate that hydrolysis of the γ phosphate of ATP may be an important step in the initiation or chain elongation or both of RNA. Thus, it is quite apparent that the polypeptides associated with the RNP complex play a vital role in all of the observed reactions described above, and these reactions may solely or partially determine the switch from the transcriptive to the replicative mode. A detailed study of the roles played by purified L, NS, and N-RNA complex should shed light on our understanding of the precise mechanism of transcription and replication of VSV.

Physical Map of VSV Genome RNA

Before discussing the structure and the possible functions of the viral polypeptides present in the RNP, it is important to take a look at the various domains of the genome RNA of VSV and point out some of their salient features and possible roles in the transcription and replication processes. It is gratifying to note that the complete nucleotide sequence of the VSV genome RNA of the Indiana serotype has now been determined. The physical map and some pertinent sequences in the genome RNA of VSV of the Indiana serotype are shown in Fig. 2. One striking feature is the junction betwen the leader template and the N gene which consists of only three A residues (117, 137, 138, 188) or four A residues in the New Jersey serotype of VSV (117). Thus, the question of how and why the RNA polymerase precisely stops at the end of the leader template without transcribing the three A residues remains an enigma. Equally perplexing is the observed attenuation of RNA synthesis at this junction (113, 223). These observations seem to indicate that the secondary structure of the RNP may play a role in the attenuation phenomenon (69, 214, 239). Alternatively, a viral protein tightly bound at that site may regulate movement of the polymerase. It is interesting to note that the virion-associated RNA polymerases of DI particles of VSV similarly synthesize precisely 46-base-long RNAs from the 3′ ends of the DI genomes and fail to extend RNA chains beyond those points in vitro (197, 200).

The second interesting feature of the nucleotide sequence of the VSV genome is the intergenic sequences. Genes N, NS, M, G, and L contain common sequences at their termini and between each other: 5′CUGUUAGUUUUUUU CAUA3′ (184). Thus, the signal for termination, polyaden-

ylation, and initiation of RNA chains may reside within this sequence. The $(U)_7$ stretch clearly signifies the region where the polymerase probably "chatters" to synthesize poly(A) (15). The dinucleotide 5'AG3', which separates the initiation site and the $(U)_7$ stretch, does not appear in mature transcripts. Moreover, the corresponding dinucleotide is AC within the intergene sequence between the NS and M genes. The role of this dinucleotide in the transcription process is unknown. The difference in the sequence of the dinucleotide at that junction has been implicated in the preferential accumulation of the readthrough product of the NS and M genes in infected cells (135). However, in VSV of the New Jersey serotype, the intergenic sequences were found to be identical to those of the Indiana serotype with no dinucleotide change in the NS-to-M gene junction (D. S. Gill and A. K. Banerjee, unpublished observations).

The third important feature of the nucleotide sequence of the VSV genome is the end of the L gene and the 5' end of the genome RNA. It contains the same termination and polyadenylation signals but lacks the signal for downstream initiation. Thus, the last 60 nucleotides, or trailer sequence, on the genome remain untranscribed in vitro. However, readthrough products containing the L mRNA linked to the complement of the end of the genome RNA have been detected in vivo (196), indicating that the entire genome is transcribed when the transcriptase is in a replicative mode.

The final interesting feature is the presence of adenine-uridine (AU)-rich sequences sequestered in specified regions of the genome RNA (113). These sequences may be the analogs of the TATA boxes which are located upstream of certain genes of eucaryotic DNA and which represent the promoter sites for RNA polymerase II. Conspicuously, there are five such boxes in the VSV genome RNA, one of which resides in the middle of the leader template and the others of which reside near the 3' ends of genes N, NS, M, and G. The sequence within the leader template curiously coincides with one of the observed binding sites of the NS protein on the genome RNA (118). Thus, it is tempting to speculate that the other AU-rich sequences may also represent binding sites of the RNA polymerase complex from which it initiates RNA transcription of downstream genes. Similar AU-rich sequences are also present in domains of the genome RNA of the New Jersey serotype of VSV (18, 83, 84).

Structure and Function of L Protein

From the complete nucleotide sequence of the L gene of VSV (Indiana serotype, Mudd-Summers strain), the amino acid sequence of the L protein has been deduced (193). The L gene spans 6,380 nucleotides and codes for a basic protein of 2,109 amino acids, with a molecular mass of 241,012 Da. The amino acid sequence of the L protein seems to be evolutionarily unique, since no significant homology to any sequence (out of 2×10^6 bases) stored in a genetic sequence data bank was observed (193). A comparison with the L proteins of other negative-strand viruses, however, awaits nucleotide sequence information from these viruses. The large size of the protein suggests that it might be multifunctional. Consistent with its size, the L gene is mapped in complementation group I of the VSV *ts* mutants, which is the group generated at the highest frequency (174). In addition, by reconstitution experiments in vitro (73, 150) and by transcription inhibition by antibodies (93), it has been unequivocally established that the L protein is one of the subunits of the RNA polymerase complex. Recently, the cloned L gene inserted into a simian virus 40 transient expression vector was expressed in COS cells. The expressed L protein complements and rescues *ts* RNA^- mutants of group I at the nonpermissive temperature, establishing that the protein is directly involved in RNA synthesis (194). Purified L protein is thermolabile (73) and appears to contain a highly reactive essential sulfhydryl or class of sulfhydryls (J. Lenard, personal communication).

The perplexing observation that neither the L nor the NS protein alone synthesizes RNA (73) made it difficult to unequivocally assign the polymerase function to either one of the polypeptides. Using reconstitution in vitro, Ongradi et al. (154) have recently shown that the L protein of VSV (New Jersey) by itself can act as a transcriptase, and the role of NS appears to be as a regulator of transcription. However, in these experiments rat liver RNase inhibitor was routinely used in the transcription reaction. Thus, the possibility cannot be ruled out that the inhibitor or a component present in it may somehow substitute for the NS function (J. Szilágyi, personal communication). Nevertheless, these results strongly suggest that the L polypeptide is the transcriptase. Recently, with purified preparations of L protein, it has been shown that the L protein and the N-RNA complex, although unable to synthesize full-length RNA, can initiate RNA chains (63). In the presence of ATP and [α-^{32}P]cytosine triphosphate, initiated transcripts such as pppAC and pppAACA are detected in vitro. By contrast, NS protein added to the N-RNA template fails to initiate oligonucleotide synthesis, nor does it stimulate oligonucleotide synthesis when added to the L protein. It appears, then, that at least the nucleotide polymerization activity is associated with the L protein. The NS protein appears to act at some step subsequent to initiation, such as chain elongation. In fact, by measuring different RNA species synthesized in the presence of limiting to saturating concentrations of NS protein, it has been shown that NS protein, indeed, plays a role in the RNA chain elongation step. The initiation function of the L protein and the chain elongation function of the NS protein seem also to correlate with the catalytic and stoichiometric requirements for these proteins, respectively (63). By using highly purified components of the RNP complex in a reconstitution reaction, an approximately 70-fold molar excess of NS protein over L protein was found to be required for optimal RNA synthesis. This is consistent with the idea that the L protein probably binds to the RNP but cannot transcribe the genome RNA due to some spatial constraint inherent in the complex. Addition of the NS protein somehow facilitates movement of the polymerase on the template. Perhaps NS protein is an RNA unwinding protein which displaces the N protein from the genome RNA in order for L protein to gain access to the genome RNA. NS protein is both highly negatively charged and phosphorylated (see below) and may be involved in displacing N protein from the template to facilitate both the entry of the L protein and its movement.

In addition to the polymerase activity of the RNP complex, four distinct virus-specific enzymatic activities have been demonstrated in the complex: capping (4), methylation (3, 180), polyadenylation (16, 68, 79, 236), and protein kinase (112, 145). None of the enzyme activities has been asssigned to any of the viral polypeptide components, except recently an L-protein-associated protein kinase activity has been detected in purified preparations of L protein (192). This protein kinase activity preferentially phosphorylates the NS protein in vitro on one or more serine residues. In a more recent study, the L-associated protein kinase activity was found to be also tightly associated with highly purified

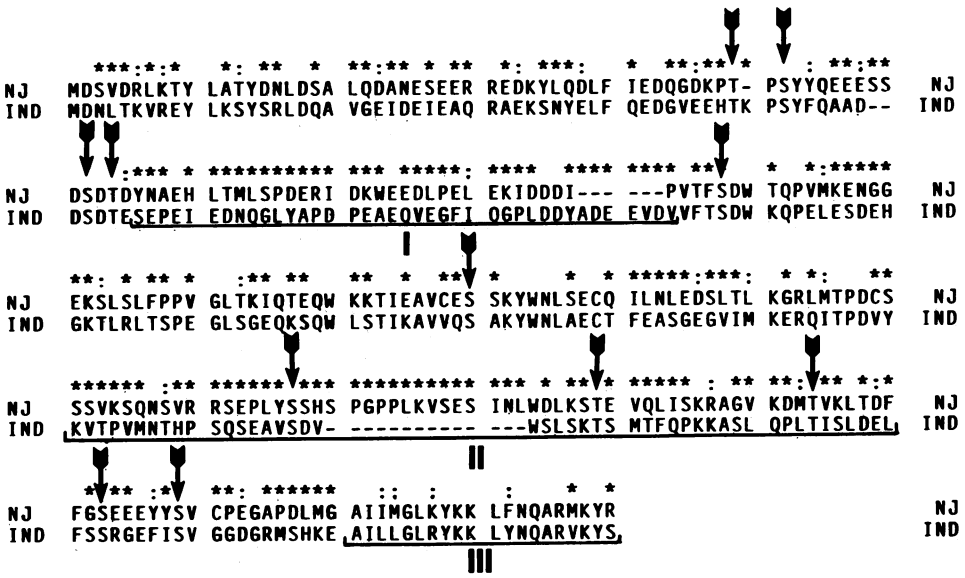

FIG. 3. Alignment of the predicted amino acid sequences (in one-letter code) of the NS protein of VSV New Jersey (NJ) and Indiana (IND) serotypes. Asterisks and semicolons are placed over the nonmatching and conserved amino acids, respectively. Domains I and II represent nonhomologous regions, and domain III represents a homologous region. The arrows indicate invariant serine and threonine residues (83).

N-RNA complex (134a). This protein kinase activity is serotype specific with regard to phosphorylation of the NS protein. Inhibition of phosphorylation of the NS protein in vitro results in abrogation of transcriptase activity by the L protein, indicating that phosphorylation of NS plays a major role in the transcription process (see below).

Capping and methylation of the mRNA products transcribed in vitro occur for a number of animal viruses that contain virion-associated RNA polymerase activities, including VSV, reovirus, and vaccinia virus (12). Only in the case of vaccinia virus have the relevant methyltransferases been purified from the virion (12). In addition, a covalently linked guanylyltransferase-guanosine monophosphate complex, an intermediate of the capping reaction, has been found in vaccinia virus (202), reovirus (201), Hela cells (230, 234), yeasts (235), and rat liver (139). In striking contrast,no such intermediate has been detected in the VSV system, although a tight noncovalent association of GDP with the NS protein has been demonstrated (61). Thus, which of the polypeptides is the capping enzyme or the methyltransferase has not been established in the VSV system. The tight coupling of capping and methyltransferase activities with the transcription reaction in vitro indicates that these activities are virus specified (1). Recently, Horikami et al. (103, 104) characterized a virus host range mutant (205), *hr*1, that is totally defective in vitro in the methylation of mRNA, although full-length polyadenylated mRNAs with 5′ termini of the form GpppA are synthesized in normal yields. A second mutant, *hr*8, is partially defective in methylation and synthesizes mRNAs in vitro with primarily GpppA and some $GpppA^m$ termini. These findings clearly establish that the virion-associated methyltransferase activities are virus encoded. Moreover, by complementation studies, Simpson et al. have suggested that the host range defect resides in the L protein (205). Thus, it appears that the VSV methyltransferase activities, and probably the capping activity, reside in the multifunctional L protein.

Recently, Hunt (109, 110) has characterized a *ts* mutant of VSV, *ts*G16(I), which synthesizes in vitro RNA containing poly(A) of aberrant size (approximately 2,500 bases). By homologous and heterologous reconstitution reactions, Hunt et al. (111) showed that the aberrant polyadenylation phenotype correlates with the presence of *ts* L protein but not with NS or M protein fraction from the mutant. Thus, the polyadenylation activity of the VSV RNP also seems to reside in the L protein.

Structure and Function of NS Protein

The amino acid sequence of the NS protein (Indiana serotype, San Juan strain), as deduced from the nucleotide sequence of a cDNA clone, reveals that the NS protein contains 265 amino acid residues with a calculated molecular mass of 29,914 Da (80, 83). Overall, the polypeptide is highly charged, with a preponderance of negatively charged amino acid residues located in the N-terminal half of the polypeptide (83). These physical characteristics, coupled with the fact that the NS molecule is heavily phosphorylated (41 serine and threonine residues of 265 total amino acids) may account for its anomalous migration in polyacrylamide gels; it migrates as a 45- to 55-kDa protein in different gel systems (131, 207). Unlike the L protein, the NS protein is highly thermostable (73); it retains its activity when heated for 2 min at 80°C (63). A better understanding of the structure of the NS protein was obtained when the amino acid sequence of the corresponding protein of VSV of the New Jersey serotype (Ogden strain) was deduced from a cDNA and compared with the amino acid sequence of VSV of the Indiana serotype (Fig. 3) (83). The two proteins are surprisingly dissimilar (30% homologous), whereas the leader RNAs, N mRNAs, M mRNAs, and G mRNAs of these two serotypes are substantially homologous: 80, 68, 62, and 51%, respectively (84). In addition, two domains (I and II, Fig. 3) were identified which are highly nonhomologous. In contrast, a third domain (III, Fig. 3), located at the C-terminal end is basic and highly conserved (90% homology). Interest-

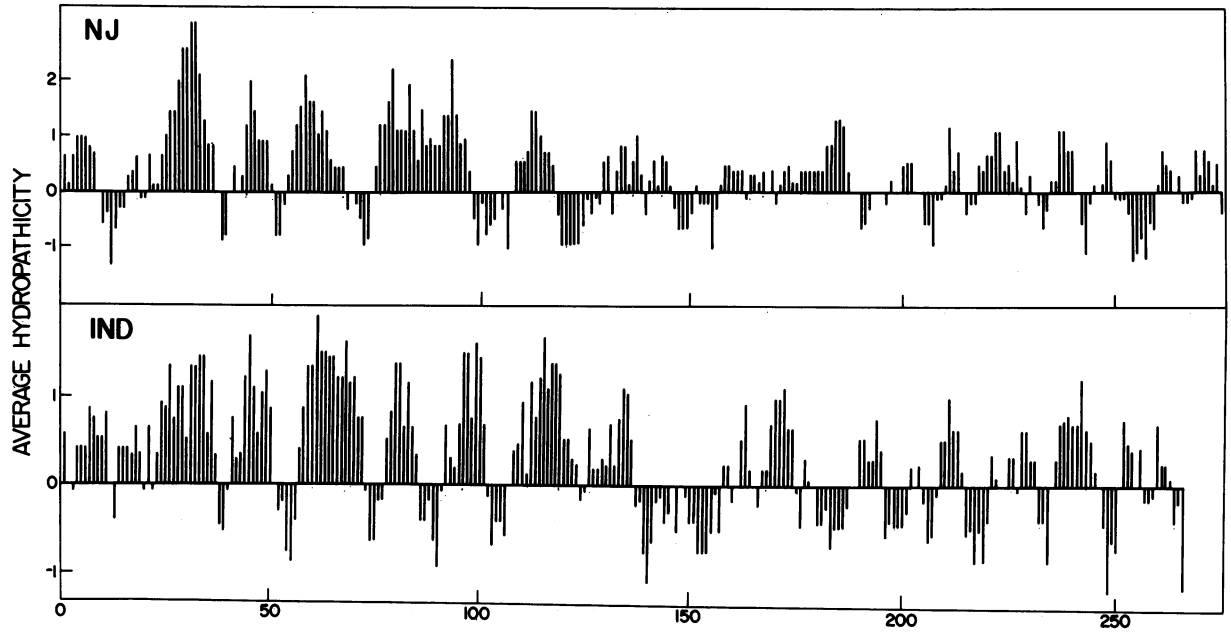

FIG. 4. Hydropathicity plot for NS protein. The distribution of hydrophilic and hydrophobic regions along the predicted amino acid sequences of NS proteins were determined (83).

ingly, despite their relatively low amino acid sequence homology, the pattern of hydrophilic and hydrophobic domains is conspicuously similar (Fig. 4), and there are 12 invariant serine and threonine residues in both NS sequences. It seems that both proteins are apparently similar structurally without having extended sequence homology, suggesting that the NS genes must have undergone divergent evolution from a common ancestor. Recently the amino acid sequence of the NS protein of the New Jersey serotype of VSV Missouri strain (Hazelhurst subtype) has been deduced from the cDNA clone (R. Elliot, personal communication). It shows a 10% difference at the amino acid level from the NS protein of the Ogden strain. However, the C-terminal domain III is totally conserved, as are the invariant serine and threonine residues. In fact, using a more rigid computer alignment program, Elliot (personal communication) has localized 18 potential phosphorylation sites that are conserved between the NS proteins of the New Jersey and Indiana serotypes. The amino acid sequence of the NS protein of the Indiana serotype of VSV Mudd-Summers strain is very similar to the San Juan strain, with only 3.8% amino acid differences (R. Lazzarini, personal communication). The amino acid sequence of the NS protein of another serologically distinct VSV, Chandipura serotype, has recently been determined from cDNA clones (P. Masters, unpublished observation). This sequence bears very little homology (23%) to the NS proteins of either the Indiana or the New Jersey serotype. Interestingly, at C-terminal domain III, 50% homology is seen.

An interesting feature of the NS genes is that each could encode a second, small basic protein (65 or 67 amino acids) originating from an internal, overlapping reading frame (Lazzarini, personal communication) similar to the phosphoprotein genes of paramyxoviruses, Sendai virus (86), measles virus (24), canine distemper virus (19), and human parainfluenza virus type 3 (130a), which have been shown to encode additional polypeptides. Despite their nucleotide sequence divergence, all five rhabdoviral NS gene sequences so far known (R. Elliott, R. Lazzarini, and P. Masters, personal communication) have the potential to encode proteins from a second reading frame which have a remarkably similar amino acid composition. However, this putative protein has not been found in the infected cells; the possible utilization of a second reading frame in the NS gene remains speculative. Herman (97) has recently detected a small NS-related protein (7,000 to 8,000 Da in molecular mass) synthesized in an in vitro protein-synthesizing extract programmed with NS mRNA. Hybrid-arrested translation demonstrated that the 7-kDa protein is translated from the 3′ one-third of the NS mRNA. Immunoprecipitation with monoclonal antibodies against the NS protein indicated that the NS protein and the 7-kDa product are translated in the same open reading frame in vitro. Thus, it seems possible that ribosomes may bind and initiate translation internally on the NS mRNA at a site located hundreds of nucleotides downstream from the capped 5′ end. The potential mode of synthesis and function of the 7-kDa protein in vivo are currently unknown.

Although the NS protein does not seem to possess any obvious enzymatic activity, it clearly plays a vital role in the transcription process. NS presumably binds to both the L protein and the N-RNA complex to carry out the elongation of RNA chains (63). Heterologous reconstitution experiments have indicated that there are specific interactions which occur between the NS and L proteins and between the NS protein and the N-RNA complex (62). For example, the N-RNA template of Indiana serotype can be transcribed in vitro by (L + NS) of the New Jersey serotype and the NS (Indiana). Thus, (L + NS) (New Jersey) can bind and initiate oligonucleotides on the heterologous N-RNA Indiana template, but there is an obligatory requirement of the homologous NS protein to carry out RNA chain elongation. These results indicate that (L + NS) (New Jersey) can bind to specific sites of the N-RNA (Indiana) but NS (New Jersey)

lacks the ability to elongate RNA chains. Thus, there must be a domain in the NS (Indiana) molecule which is absent in NS (New Jersey) and which confers specificity towards the homologous N-RNA (Indiana). When the inverse reconstitution experiments were performed, a different specificity was observed (62). The N-RNA (New Jersey) template can be transcribed by L (New Jersey) and NS (Indiana) but not by (L + NS) (Indiana) and NS (New Jersey). These results strongly suggest that L (Indiana) lacks a domain with which NS (New Jersey) interacts, whereas L (New Jersey) contains a domain with which NS (Indiana) can interact. If the latter is true, there must be a common domain in the NS protein in both serotypes which interacts with L (New Jersey).

With the availability of the primary structure of the NS protein, it now has become possible by genetic engineering to map the domains in the polypeptide which are involved in various functions of the NS molecule. Recently, by inserting a full-length NS gene of the New Jersey serotype into an SP6 transcription vector, it was possible to transcribe full-length NS mRNA in vitro and then translate it into NS protein in a cell-free rabbit reticulocyte protein-synthesizing system (84a). This translated NS protein associates functionally with the homologous L protein and the N-RNA complex in a reconstituted transcription reaction, resulting in the synthesis of leader RNA and the five VSV mRNA species. Removal from the inserted gene of the sequence encoding the highly conserved C-terminal end (domain III) of the NS protein results in the synthesis of a truncated mRNA and its corresponding protein. This protein still retains most of the transcriptase activity (75%) when reconstituted with L protein and N-RNA, but it lacks the capacity to bind tightly to the N-RNA template. These results clearly indicate that domain III (Fig. 3) may be involved in tight binding to the N-RNA complex, but a truncated NS protein, in the presence of L protein, binds adequately to transcribe in vitro. Further deletion of the NS gene defined a domain between amino acid residues 213 and 247 of the NS protein that is found to be essential for in vitro transcription. Additional genetic manipulation of the NS gene is likely to reveal domains which are involved in transcription, chain elongation, replication, and phosphorylation.

The studies related to the phosphorylated state of the NS protein as well as to the role of NS phosphorylation in transcription and replication have been accumulating for several years. There are a number of reports which indicate that inhibition of in vitro phosphorylation of NS protein by various compounds results in the inhibition of VSV RNA synthesis in vitro (48, 119, 120, 192, 206, 236, 244). However, the cogent question still remains unanswered as to the precise location of the functional phosphorylation sites within the NS molecule. Clinton et al. (49) demonstrated that two forms of the NS protein, NS1 and the more highly phosphorylated NS2 form, can be resolved in sodium dodecyl sulfate-polyacrylamide gels containing urea. A comparison of chymotryptic peptides indicated that the same two phosphorylated forms are found in virions as well as in the cytoplasm of infected cells. Dephosphorylation of NS by cellular phosphatases results in conversion into the NS1 species. Both phosphorylated species contain phosphothreonine and phosphoserine, with the latter being the principal phosphorylated amino acid. Hsu et al. (105, 107, 108) have established that within these NS1 and NS2 species there are subsets of as many as 21 phosphorylated serine and threonine residues. In addition, the NS1 species is more resistant to bacterial alkaline phosphatase than NS2. More recently, Hsu and Kingsbury (106), Bell and Prevec (22), and Marnell and Summers (133) have demonstrated that the constitutively phosphorylated sites in the NS molecule reside in the amino-terminal region of the molecule, between residues 35 and 106. Hsu and Kingsbury (106) concluded that this domain is embedded within the tertiary structure of the molecule such that the phosphoamino acids are resistant to exogenous phosphatase action. In contrast, the amino acid residues that are phosphorylated secondarily reside in a more exposed region of the molecule. The latter phosphorylation renders the NS molecule active in transcription.

Kingsford and Emerson (120) studied the role of these variously phosphorylated NS species in the transcription process, using an in vitro reconstituted system. In their studies the NS protein was fractionated into a less phosphorylated NSI and a more highly phosphorylated NSII fraction by diethylaminoethyl cellulose chromatography. NSI and NSII each could be further separated individually into the conventional NS1 and NS2 subspecies. By contrast, cytoplasmic preparations of NS contained one phosphorylated species which eluted from the diethylaminoethyl column in a different salt concentration than the virion NSI. When in vitro reconstitution experiments were performed, NSI and cell NS exhibited little activity, whereas NSII was very active in the same system. Addition of NSI or cell NS to a transcription system containing NSII resulted in even higher levels of activity. These results indicate that various NS species have different levels of activity and that intracellular NS (cell NS) is transcriptionally inactive. Kingsbury et al. (119) demonstrated that enzymatic dephosphorylation of the NS2 species within the RNP reduces in vitro transcriptase activity up to 80% . Rephosphorylation by the protein kinase in the virion does not restore the native phosphorylated species, accounting for the irreversibility of transcriptase inactivation. From the above studies it is reasonable to conclude that phosphate residues located at specific sites in NS potentiate the performance of the protein in transcription. However, the precise roles of the NS1 and NS2 subspecies in the transcription process still remain unclear.

Recently, Masters and Banerjee (134a) investigated the phosphorylated state of the virion and cytoplasmic forms of NS and compared their roles in transcription in vitro. These studies revealed that the NS1 and NS2 subspecies are differentially recognized by viral and cellular protein kinases and show contrasting sensitivities to phosphatase action. The cellular protein kinases are mostly active in phosphorylating the NS1 form of the protein. The resulting hyperphosphorylation of NS1 is essentially irreversible. In contrast, the L-protein-associated protein kinase (192) preferentially phosphorylates the NS2 form of the protein in the presence of N-RNA complex. Both in vitro- and in vivo-phosphorylated NS2 are totally sensitive to the action of one or more cellular phosphatases. These results indicate that the two forms of NS are conformationally different, and since both are needed for transcription, each may play a specific role in the transcription process. In the same study, the cytoplasmic form of NS protein was found to be fully capable of binding to the virion N-RNA template, and the pelleted complex, in conjunction with the L protein, participated in the synthesis of the leader RNA and the five VSV mRNA species. Preferential binding of NS1 to N-RNA occurred under these conditions. However, the trace amount of NS2 associated with the complex was the form of the protein that was almost exclusively phosphorylated, suggesting that it is required in catalytic amounts and has a high affinity for the L protein and the N-RNA complex. In

the light of the previous report that the NS protein is needed in high molar excess in the transcription reaction (63), the above results suggest that in infected cells NS1 is the species required in nearly stoichiometric amounts with the template N protein complex. The dynamically phosphorylated-dephosphorylated NS2 possibly serves a catalytic role in VSV RNA synthesis. It is tempting to speculate that the NS1 form acts as a highly acidic protein reacting with the N protein to serve as an RNA unwinding protein (63). The NS2 protein, by virtue of being phosphorylated at specific sites by the L protein, may bind tightly with the L protein and initiate RNA synthesis. Detailed study along these lines is clearly needed to unravel how the conformational states of the NS protein change with phosphorylation and to understand the precise function of the multimeric phosphorylated NS species in the VSV transcription process.

Structure and Function of the N Protein-RNA Complex

The nucleocapsid protein (N), which is tightly associated with the negative-strand genome RNA, forms the required template for both transcription and replication of the genome. This polypeptide, although an integral part of the RNA polymerase-template complex, has not been studied in detail due to the unavailability of the N protein in its native form in solution; it rapidly aggregates when removed from the template (29). N protein expressed in cells from a molecularly cloned N gene is found also to be aggregated with cellular RNAs (209), indicating that N protein is an RNA-binding protein. However, N protein from intracellular VSV nucleocapsids has recently been solubilized, and its role in specific binding to leader RNA in vitro as well as its direct role in replication have been demonstrated (see below). The N protein accounts for 90.7% of the protein mass of purified nucleocapsids and is represented at 1,258 copies per nucleocapsid (222). Based on this and on the number of nucleotides in the VSV genome RNA, an approximate value of 9 is determined for the number of nucleotide bases per N protein monomer in the repeating unit of the nucleocapsid strand. Interestingly, this value correlates well with the properties of the 5′-terminal sequences of VSV leader RNAs, which have a high affinity for N protein and in which Blumberg et al. (31) identified a largely conserved 18-residue sequence containing a high incidence of adenosine. This sequence has been implicated as the site where initiation of nucleocapsid assembly occurs in vivo. From biochemical experiments it has been shown that the genome RNA within the nucleocapsid is resistant to RNase action even during transcription in vitro (38, 94, 208). The mechanism by which N protein interacts with the (L + NS) complex to allow the latter to gain access to the template RNA remains an enigma. It is noteworthy that highly purified N-RNA complex obtained from virions remains transcriptionally competent even after repeated banding in CsCl followed by dialysis at room temperature for days (224), indicating that the N protein-RNA is a highly stable complex. Although it is possible to obtain N-RNA complex virtually free of L and NS proteins (as judged by silver staining), it still retains traces of these proteins to be able to initiate infection when microinjected into cells (224).

The complete amino acid sequence of the N protein has been deduced from cDNA clones for both the Indiana (80) and the New Jersey (18) serotypes of VSV. Both proteins contain 422 amino acid residues and have approximately 68% identity with each other. An overall homology of >80% of the amino acid sequences is observed when conservative replacements of the amino acids are considered. Moreover, the hydrophilic domains in both proteins are very similarly sequestered. Consistent with this observation, polyclonal antibodies raised against N proteins of either serotype cross-react (64). In contrast, a monoclonal antibody raised against N protein of VSV (Indiana) does not cross-react with the N protein of VSV (New Jersey) and inhibits in vitro transcription of the homologous RNP (64). Presumably this monoclonal antibody is directed toward an antigenic site involved in transcription and includes one or more of the amino acid residues which differ between the two proteins. It is not known which domain of the N protein interacts with the (L + NS) complex during transcription or binds to the leader RNA to initiate replication. Future studies with recombinant DNA technology will be essential to establish the function of the N protein in transcription and to relate structure to function.

Regulation of RNA Synthesis: Role of M Protein

Since purified RNP devoid of the G and M proteins is active in in vitro transcription (26), it is generally assumed that these proteins have no role in the RNA synthetic process. However, early data with mutants indicated that a lesion in the M protein (complementation group III) increases viral RNA synthesis. Clinton et al. (50) and Martinet et al. (134) directly demonstrated that *ts* mutants in this group indeed produce a large excess of RNA at the nonpermissive temperature. Quantitation of individual mRNA species indicated that M protein acts as a direct inhibitor of transcription as well as an attenuator of sequential transcription (37, 56).

The observation by Perrault and Kingsbury (165) that purified VSV contains a serotype-specific inhibitor for in vitro transcription, coupled with the finding that certain polyanions, in particular poly(L-glutamic acid), reverse the transcriptase inhibitor activity at high concentrations of virion, led Carroll and Wagner (36) to propose and demonstrate a direct role of M protein in the in vitro transcription reaction. These results were confirmed by Wilson and Lenard (241), who showed an ionic strength-dependent interaction of M protein with the RNP in vitro, leading to inhibition of RNA synthesis. This interaction is integrally related to the temperature-sensitive phenotype of group III mutants. Interaction of M protein with the RNP resulted in a striking morphological change in the RNP as seen in the electron microscope (65, 152). The native helical structure of the RNP is converted into a highly condensed structure by the addition of purified M protein at low ionic strength. Analysis of the RNA products synthesized under conditions of M protein inhibition demonstrated that only a few triphosphate-initiated RNAs (11 to 14 bases), corresponding to the 5′ terminus of the N-mRNA, are synthesized in vitro (65, 171, 172). Moreover, initiation on the leader template is significantly increased at low ionic strength, suggesting that the M protein interaction possibly occurs at the chain elongation step of RNA synthesis (65). The M protein, especially at its N-terminal domain, is a highly basic protein, as predicted from the deduced amino acid sequence obtained from cDNA clones (185). Whether the basic domain plays a role in the interaction with the N-RNA template is still unclear. It is noteworthy that, from sequence data obtained by primer extension on the genome RNA, Gopalakrishna and Lenard (87) have found virtually no change in the N-terminal basic portion of the M protein of four *ts* mutants

belonging to group III. The mutations in these viruses are so widely distributed along the polypeptide chain that they are difficult to interpret in terms of a specific nucleocapsid-binding site.

Recently, Wagner and his co-workers (155, 156, 246) have raised monoclonal antibodies against the M protein to study the role of M protein in viral transcription in vitro. Monoclonal antibodies to three distinct antigenic determinants affected in vitro transcription by wild-type RNP/M cores (RNP with M protein attached) in widely divergent ways. The inhibition of transcription by such an RNP is reversed by antibody to epitope 1. In striking contrast, antibody to epitopes 2 and 3 not only failed to reverse the transcription inhibitory activity of isolated M protein, but increased it in a reconstituted system. Epitope 1 is not present in the M protein of the mutant *ts*23 but is present in a revertant in which transcription inhibition is restored. It seems likely that mutations in the VSV M protein gene occur at different regions, and the resulting difference in phenotype may be due to conformational changes in M protein that alter its binding affinity to RNP cores. This supports the conclusions drawn by Gopalakrishna and Lenard (87) in their studies to determine the amino acid sequence of the M proteins of *ts* mutants. Insertion of the M gene into an expression vector (156) and subsequent genetic manipulation to synthesize truncated proteins should help map more definitively the nucleocapsid-binding domain by using the available battery of monoclonal antibodies.

REPLICATION OF RHABDOVIRUSES

For all negative-strand RNA viruses, including rhabdoviruses, infection begins with the synthesis of the viral mRNAs by the virion-associated RNA polymerase of the infecting virus. This RNA-synthetic step is commonly called "primary transcription." The translation of these viral transcripts leads to the onset of genome replication. Continued protein synthesis is required for the maintenance of genome replication, since addition of protein synthesis inhibitors, such as cycloheximide, blocks replication but not primary transcription (160, 240). Unlike the template for primary transcription, which is the negative-strand RNP complex, the template for genome replication is the positive-strand RNP complex. The conversion of negative-strand RNP to positive-strand RNP is presumably mediated by a switch of the RNA polymerase from the transcriptive to the replicative mode. Since free positive-strand genome-length RNA is not found in infected cells, the concomitant association of the N protein with the growing positive-strand genome RNA seems to be a plausible mechanism of positive-strand nucleocapsid formation (208). The newly synthesized positive-strand RNP then serves as the template for replication, and amplification of negative-strand RNP ensues. Since the positive-strand RNP does not contain signals for transcription, this serves exclusively as a template for replication. Again, the N protein serves a vital role in the assembly of the full-length negative-strand genome RNA into RNP. Thus, a possible explanation for the effect of cycloheximide is that genome replication is coupled to the continued synthesis of a viral component (such as the N protein) which is required in stoichiometric rather than catalytic amounts. In contrast, genome transcription requires catalytic amounts of the viral polymerase (63). Transcription from the amplified negative-strand RNP (commonly called "secondary transcription") then leads to the amplified synthesis of all viral proteins needed for subsequent maturation of the virus.

Unlike the transcription step in the rhabdoviral life cycle, the replication step has not been studied in great detail. However, in the recent past an impressive series of publications, including several reporting in vitro replication systems, have specifically dealt with the replication process of VSV.

Consistent with the above replication scheme, Hill et al. (101), Simonsen et al. (204), and Rubio et al. (191) isolated and characterized VSV replicating complexes from infected cells by using Renografin (E. R. Squibb & Sons, Princeton, N.J.) gradients. A majority of nascent RNA molecules of less than half genome size in the mixture of replicative RNPs are complementary to the 3′ end of the VSV positive strand, i.e., the L-protein cistron. These nascent RNA species rapidly become associated with the N, L, and NS proteins, are RNase resistant, and thus are presumed to be within RNP complexes. Moreover, the formation of these RNPs depends on continued protein synthesis, indicating that they are indeed replicating complexes. Simonsen et al. (203) further demonstrated that, early in infection, positive-strand RNA composes 40% of the full-length RNA synthesized, whereas late in infection only 15 to 20% of the 42S RNA synthesized is positive strand. The rate of synthesis of the positive strand remains constant throughout infection. The precise structure of the replicating RNP, however, remains elusive. Based on electron microscopic analyses, Naeve et al. (147, 148) suggested that the replicating RNP exists in a complex structure, possibly in a circular form. A plausible advantage of using a circular template for replication would be to cycle a single enzyme complex over a single template several times. This hypothesis must remain tentative until additional experiments are carried out.

Interaction of N Protein with Leader RNA

One of the prerequisites of the replication scheme described above is the encapsidation by N protein of the growing full-length positive- or minus-strand genome RNA. That the leader RNA is the first product to be synthesized in vitro during transcription (52) led Blumberg et al. (31) to formulate a model of genome replication in which the N protein acts as a modulator of genome transcription and replication. In this model, the synthesis of VSV leader RNA is terminated at an attenuation signal located approximately 50 nucleotides downstream from the 3′ end of the genome template. The viral N protein modulates transcription and replication by its ability to bind to the nascent leader RNA, and it acts as an antiterminator of leader RNA, resulting in the synthesis of full-length positive-strand RNP. The N protein may also be an antiterminator at each intergenic junction. Once the positive-strand RNP is formed, replication proceeds with the synthesis of negative leader RNA (RNA identical to the 5′ end of the genome RNA), which binds nascent N protein to form a negative-strand RNP. In fact, Leppert et al. (128, 129) have shown that both positive and negative leader RNAs are present in VSV-infected cells. Moreover, these workers have shown that negative leader RNA synthesis increases in the presence of cycloheximide, which blocks genome RNA synthesis. Thus, it seems that continuous protein synthesis is not required for initiation of genome RNA synthesis, but is required for the synthesis of completed genomes. A possible explanation for these observations is that, in the absence of viral protein synthesis, the polymerase synthesizes a 47-nucleotide RNA on both genome (negative-strand) and antigenome (positive-strand)

templates and then terminates the RNA chains at strong stop signals present at similar points on both templates. The function of this stop signal on the antigenome template is to ensure that negative-strand genome RNA is not made in the absence of viral protein synthesis. This situation may be similar to the VSV DI particle RNA polymerase reaction in which only negative leader RNA is synthesized in vitro (197, 200). However, the nature of the putative strong stops on the genome or antigenome RNA remains poorly understood.

Direct evidence that the N protein indeed interacts with the leader RNA came when it was shown that an 18S N protein-leader RNA complex is formed in VSV-infected cells, where the leader RNA within the complex is resistant to RNase (28, 30). The majority of positive leader RNA found in infected cells late in infection appears to be present not as free RNA but as a complex with viral protein. The existence of leader RNA complexed with the N protein, however, does not conclusively demonstrate the antitermination property of the N protein. Nevertheless, it shows that the leader template is the preferred target for the N protein to initiate the process of encapsidation. Using purified soluble N protein, Blumberg et al. (28) were able to demonstrate that it selectively assembles leader RNAs over other viral transcripts. By using end-labeled leader RNA fragments, it was shown that the leader RNA assembly starts within the first 14 nucleotides from the 5′ end. In all of the known sequences of positive and negative VSV leader RNAs (85), a high percentage of A residues is found in the leader RNA, with some of them located at every third base (5′-ppACGAANACNANNAAACCA . . . 3′). Based on these observations, Blumberg et al. (28) suggested that these A residues are important for encapsidation of leader RNA with the N protein. They pointed out that a similar triplet phase repeat of G residues present at the nucleation origin of the tobacco mosaic virus genome RNA has been strongly implicated in the assembly of genome RNA with the coat protein.

Since the 12-kilobase VSV genome is encapsidated by about 2,000 N proteins (although recently a value of 1,258 for N protein has been shown; see reference 222), while the 47-nucleotide leader-nucleocapsid structures appear to contain eight monomers of N protein, it appears that each N protein monomer binds to six nucleotides. It is postulated that enough information would be present in the first 12 bases of the nascent leader RNA strand to facilitate assembly of the first two N proteins. Further assembly is maintained by cooperative binding of the N protein (28). In a recent report, however, VSV isolated from persistently infected L cells appeared to lack such A triplets in the leader sequence (243), suggesting that other features in the leader RNA may also play a role in the encapsidation process. It is interesting to note that the leader RNAs and the N proteins of VSV of the Indiana and New Jersey serotypes are highly homologous (18). Thus, it is possible that in a mixed infection heterologous RNP would be formed since the signals for encapsidation are present in both leader RNAs. In fact, in a mixed infection with DI particles of the two serotypes, heterologous RNPs are indeed formed, indicating that common information is involved in the encapsidation of leader RNAs of both serotypes (45).

The direct involvement of the N protein in the viral replicative process, discussed above, gained further support from the development of an in vitro replication system in which encapsidation of the nascent RNA chains by the N protein was directly demonstrated.

In Vitro Systems for Study of Rhabdoviral Genome Replication

In the recent past, several laboratories have initiated studies to develop suitable in vitro VSV replication systems to examine the roles and requirements of viral proteins in the replication process. These systems have allowed us to take a clearer look into the complex nature of VSV replication. Batt-Humphries et al. (20) originally described an in vitro replication system in which total cell extracts from VSV-infected HeLa cells were used to assay for ability to synthesize genome-length RNA. Subsequently, Hill et al. (100) optimized the HeLa cell S10 extract for transcription and replication and demonstrated replication and assembly of VSV genome-length RNA into nucleocapsids containing N protein. The in vitro synthesized RNPs, which represented 1 to 2% of the total RNA product, were RNase resistant and had the same buoyant density in CsCl as virion nucleocapsids. Moreover, the in vitro replication reaction was dependent on protein synthesis, or at least on a pool of VSV RNP proteins. Both full-length positive- and negative-strand RNAs were synthesized in the same ratio as seen in vivo, indicating that probably both positive- and negative-strand RNPs were present in the S10 extract. Similar results were obtained when viral RNPs isolated from VSV-infected cells were allowed to transcribe and translate mRNAs in the presence of uninfected HeLa cell S10 extract (82). Antibody raised against both VSV NS and N proteins inhibited in vitro RNA replication (102). Interestingly, anti-L antibody stimulated replication twofold, although the synthesis of mRNA was inhibited. The simplest explanation for these findings would be that newly synthesized NS and N proteins are both required for ongoing replication both in vivo and in vitro. This is also consistent with the observation that VSV RNA replication is dependent upon ongoing protein synthesis, or at least the presence of certain VSV-specific proteins. The reason for the stimulation of replication by the L antibody, however, remains unclear.

Condra and Lazzarini (57) developed a permeable cell system to study replication of VSV. Using lysolecithin permeabilization of VSV-infected BHK-21 cells, they have shown that ribonucleoside triphosphates are incorporated into the transcribing and replicating RNAs, which closely resemble those found in vivo. In addition, full-length nucleocapsids of both polarities are formed. On the other hand, Peluso and Moyer (161) prepared permeabilized VSV-infected BHK-21 cells from which a cell-free supernatant fraction (free of cellular DNA) was obtained that initiates replication in vitro; VSV RNP was used as the transcribing complex. The level of 42S RNA synthesis in vitro is considerably higher (6 to 13% of the total RNA synthesized) than that of previously reported in vitro systems. To study initiation of RNA replication, Peluso and Moyer (161) used a VSV DI particle (MS-T) which can transcribe in vitro only a 46-nucleotide negative leader RNA. When this DI RNP is added to VSV-infected cell extract, synthesis of full-length 19S DI RNA is observed, indicating that both the initiation of RNA replication and its elongation must occur in vitro.

Since only positive-strand MS-T DI RNP is synthesized in vitro, it appears that the extract lacks one or more of the proteins necessary to synthesize the negative-strand RNP. Interestingly, inhibition of VSV replication in vitro occurs when both VSV and MS-T DI are added to the in vitro replication system, demonstrating that an interference phenomenon is operational in this system. Since these extracts can support the synthesis of VSV proteins, RNA replication

is not dependent on de novo protein synthesis but uses the preformed soluble proteins present in the infected cell at the time the extract is prepared. Using monoclonal antibodies, Peluso and Moyer (162) demonstrated that a putative N-NS protein complex, and not the N protein alone, is directly involved in the replication process. This complex is present in infected cells and is involved in the binding to nascent replicative RNA. Moreover, following encapsidation, the NS protein appears to become dissociated since no coordinate binding of the NS and N proteins is observed in vitro. Similar complexes of the N and NS proteins in the soluble cytoplasmic fraction of VSV-infected cells are also detected by immunoprecipitation with monospecific anti-NS antisera (21). These observed roles of NS protein in VSV replication are consistent with the previous studies on VSV mutants which established clearly a replicative role of NS protein in the life cycle of VSV (130). It is interesting to note that when the highly conserved C-terminal portion (Fig. 3) of the NS protein is removed, the residual protein, although it supports transcription in vitro (when added to N-RNA complex in the presence of L protein), lacks a strong binding affinity for the N-RNA complex (84a). Thus, the C-terminal domain of the NS protein may have a role in replication through its ability to bind to N protein.

Wertz and co-workers (60, 157) have developed a highly purified in vitro replication system which produced much of the answer as to the roles and requirements of VSV proteins in the replication process. The major components of the system are (i) an mRNA-dependent rabbit reticulocyte lysate to carry out cell-free protein synthesis; (ii) the five VSV mRNAs, or separately isolated individual VSV mRNAs, to program VSV-specific protein synthesis; and (iii) nucleocapsids containing positive- and negative-strand genome RNA. Approximately 200 pmol of protein per ml is synthesized, and all of the newly synthesized full-length VSV RNA, which represents 2 to 5% of the total RNA product synthesized in vitro, bands in CsCl at the position of the nucleocapsid and is RNase resistant. The molar ratio of the nucleocapsid-associated, newly synthesized proteins is 2:350:1,000:10 (L/NS/N/M), and >90% of the newly synthesized NS protein associated with the nucleocapsids in vitro is of the NS2 subspecies. In a similar series of experiments, Wertz (238) demonstrated that the in vitro replication system also supports synthesis of full-length VSV DI particle RNA and carries out the encapsidation process in vitro. In this system, the transition from synthesis of only the DI negative leader RNA, a transcription event, to the replication of full-length DI RNA is made as a function of viral protein synthesis. Thus, the in vitro transcription-translation-replication system provides an opportunity to dissect the requirements for replication in vitro.

Using individual VSV mRNAs purified by cDNA hybridization, Patton et al. (157, 158) showed that the VSV N protein, alone, results in the replication of genome RNA by both intracellular RNP from VSV DI and virion-derived VSV RNP. Neither the NS nor the M protein supports RNA replication. Presynthesized N protein is inefficient in replicating genome RNA in vitro. It was concluded that the newly synthesized N protein allows initiation and elongation, as well as encapsidation, of genome-length RNA. Recently, Wertz and co-workers (M. Howard, N. Davis, J. Patton, and G. Wertz, *in* B. W. J. Mahy and D. Kolakofsky, ed., *The Biology of Negative Strand Viruses*, in press) have shown that the NS protein also profoundly affects the ability of the N protein to function in supporting replication. The reticulocyte lysate translation system programmed with all five VSV mRNAs contained complexes of the N and NS proteins which were immunoprecipitated with both anti-N and anti-NS monoclonal antibodies. Moreover, the molar ratio of N/NS was found to be critical in determining the optimal levels of replication. Based on these findings and the findings of Peluso and Moyer (162), Wertz and co-workers (in press) have proposed a model in which the NS protein regulates replication by controlling the availability of N protein. In this model the N protein alone can support replication if the N protein is present in low concentration. At higher concentration, the N protein aggregates and becomes incompetent in replication unless the NS protein is present to prevent it from aggregation and maintains it in a replication-competent form. Critical to this complex formation is the N protein/NS protein concentration ratio. When the molar amount of NS protein exceeds that of N protein, replication is inhibited. Thus, the NS protein may be the factor that controls the balance between VSV RNA replication and transcription, and it may do so by controlling the availability of N protein to support replication. In support of this model, Arnheiter et al. (5) have recently shown that monoclonal antibodies to the VSV N protein can differentiate between two forms of the N protein, both in vivo and in vitro. Antibody 1 binds to nucleocapsids and to the pool of free (unbound) N protein and inhibits both transcription and replication in vitro. When microinjected into cells, it protects the cells against VSV. In contrast, antibody 2 binds poorly to nucleocapsids, and it does not inhibit transcription, but it inhibits replication in vitro. When microinjected into cells, it binds selectively to free N protein and delays the appearance of progeny virus. In addition, antibody 1 can precipitate both N and NS proteins, whereas antibody 2 precipitates only free N from a translation mixture. These results strongly suggest that the N protein is present in different forms (possibly free and bound to the NS protein) in infected cells and that the availability of the N protein is one of the factors controlling RNA synthesis. Continued study along this line should shed light on the precise roles of the N protein and of the different phosphorylated forms of the NS protein in the VSV replication process.

In Vitro Replication in the Absence of N-Protein Synthesis: VSV *polR* Mutants

The in vitro replication systems described above included in the reaction mixture either de novo synthesized or preformed N protein to facilitate replication and packaging of newly synthesized full-length genome RNA into an RNP complex. However, the precise mechanism by which the nascent or preformed N protein switches the virion polymerase from the transcriptive to the replicative mode remains unclear. The question remains whether the newly synthesized leader RNA requires concomitant binding with the N protein to act as an antiterminator or whether full-length genome RNA or readthrough RNA products can be synthesized without added N protein.

Testa et al. (220) and Chinchar et al. (44) demonstrated that virion RNP, preinitiated with ATP and cytosine triphosphate, may synthesize either readthrough or full-length positive-strand genome RNA when in vitro RNA synthesis is subsequently allowed to proceed in the presence of AMPPNHP, an ATP analog which contains a nonhydrolyzable β-γ phosphate bond. Similarly, Chanda et al. (42) showed that under the same conditions various VSV DI particles of different genome sizes, which normally synthe-

size only a 46-nucleotide negative leader RNA in vitro, synthesize genome-length positive-strand RNA in vitro. The requirement for a hydrolyzable β-γ ATP bond seems to be important for the initiation of RNA synthesis, whereas chain elongation can proceed in the presence of AMPPNHP, leading to full-length RNA synthesis. These results imply that the virion-associated RNA polymerase may change to the replicative mode without the requirement of added N protein. Moreover, phosphorylation appears to play an important role in the observed in vitro replication process. It is possible that the interaction of specifically phosphorylated NS protein with the L protein leads to the formation of an enzyme complex capable of initiating replication on the template RNP. Coupled with the observation that the exogenous presence of N protein facilitates replication, it may be envisaged that an interaction of the N, NS, and L proteins is crucial to attaining the replicative mode of the RNA polymerase. It is noteworthy that readthrough or full-length RNA products have also been synthesized in vitro by purified RNP in the presence of thio-ATP (99) and inosine triphosphate (41, 217). The mechanism by which these compounds alter the specificity of the virion RNA polymerase enzyme is not clear.

Perrault and co-workers isolated a novel VSV mutant, denoted *polR*, and found that both this virus and DI particles generated from it efficiently read through the leader termination site under standard conditions of in vitro RNA synthesis (166, 167). *polR1* virus was obtained after a selection procedure involving several cycles of heat inactivation of wild-type virus. The mutant virus gave rise to a much higher proportion of readthrough transcripts than wild type (80 versus ~10%). Moreover, *polR* mutants synthesize RNA relatively efficiently in the presence of AMPPNHP (168) compared with the wild type. This requirement for ATP possibly reflects some initiation or preinitiation event required for synthesis beginning at the 3′ end of the template. Transcription reconstitution experiments with purified RNP template and enzyme fractions containing the L and NS proteins from wild-type and mutant virus revealed that the N protein moiety of the RNP template is responsible for the observed readthrough phenomenon (164). These results indicate that readthrough can occur solely as a result of an alteration in the N protein and without concurrent nucleocapsid assembly. Perrault et al. (164) have subsequently shown that the N proteins of the wild-type and the mutant viruses contain differently charged isoelectric species, indicating that this protein is post-translationally modified. Based on these results, Perrault et al. (164, 169) proposed a model in which preexisting assembled RNP template structures (and not assembly of nascent leaders) determine whether readthrough of the leader termination site can occur. Inherent in this model is the suggestion that wild-type virus or wild-type DI particles package RNPs whose structures promote termination at the leader RNA sites under normal transcription conditions in vitro. However, in vivo these "transcription RNPs" are modified to "replication RNPs" which will promote replication by readthrough of both positive and negative leader termination sites. Such modification of RNPs is presumably mediated by alteration of the N protein associated with the RNP. Subsequent packaging of the nascent RNA with the N protein proceeds by interaction with the growing readthrough RNA. An important consideration for this model is to establish the precise nature of the modifications of the N protein and the pathways leading to these modifications. Perhaps one can reconcile the two models of replication if the modification of the N protein somehow governs its ability to interact with NS.

CONCLUSIONS

In this review I have attempted a comprehensive discussion of two important steps in the rhabdoviral life cycle, i.e., transcription and replication. A thorough understanding of these two complex processes should help us understand the mechanism by which the viruses in this family express their genes. As anticipated in the Introduction, the studies on transcription and replication of VSV occupy virtually this entire review due to the continued use of VSV as a model rhabdovirus in many laboratories. However, it would be presumptuous to equate the results obtained from the studies on VSV to all of the rhabdoviruses of mammals and fish, let alone those of plants. It is becoming increasingly clear that many rhabdoviruses behave quite differently from each other, specifically with regard to the existence of different modes of RNA synthesis, the presence of additional genetic elements, and phosphorylated proteins. Thus, continued study of different groups of rhabdoviruses is important to gain a clearer picture of the evolution and mode of gene expression of these viruses.

Even in the best case, the thoroughly studied VSV, the precise mechanism of transcription and replication is not yet fully understood. As described above, a seemingly straightforward in vitro transcription system has thus far failed to show unequivocally how mRNAs are produced from the genome template. Particularly perplexing are the phenomenon of attenuation and the inability to identify and characterize directly the proteins mediating the capping, methylation, and polyadenylation of mRNAs. Thus, much remains to be learned regarding the functions of the three polypeptides which constitute the transcribing RNP, i.e., the L, NS, and N proteins. By molecular cloning and subsequent expression of their genes, one should be able to reconstitute transcription in vitro and, by deletion mapping and site-specific mutagenesis, probe into various domains of these proteins and eventually relate these domains to various functions.

Similarly, an in vitro replication system has provided insight into understanding the roles of virus proteins involved in this process. However, the precise mechanisms by which the virion polymerase switches from a transcriptive to a replicative mode remain unclear. Does the interaction of the nascent N protein with the leader template provide the antitermination signal, or does a specific replication complex of the N, NS, and L protein interact with the N-RNA template, leading to replication? Alternatively, the N protein within the RNP may solely regulate transcription and replication as suggested by Perrault et al. (164). To answer these important questions, one needs to develop an in vitro replication-reconstitution system, using the N-RNA complex and either purified or nascent L, NS, and N proteins to initiate both transcription and replication in vitro. Recombinant DNA technology should prove to be a powerful tool to study this process. There are some observations which indicate that host factors may play roles in the VSV transcription and replication processes (175, 210, 211). Recently, a host protein (La) has been shown to specifically bind to the leader RNA during replication of VSV (126, 242). The precise role of this protein or any host protein in VSV replication remains to be determined. Thus, the VSV transcription-replication system still remains an interesting system for which challenging questions remain unresolved, and

important new findings are bound to emerge in the future which will help us to understand VSV and other negative-strand RNA viruses.

ACKNOWLEDGMENTS

I am grateful to Aaron J. Shatkin and Paul S. Masters for reading the manuscript and for their valuable comments. I also thank my numerous colleagues working in rhabdovirus gene expression for supplying their reprints and communicating their unpublished results. My sincere appreciation is due to Josephine Marseglia for her excellent secretarial assistance.

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