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Nucleotide Sequence and Host La Protein Interactions of Rabies Virus Leader RNA

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Rabies virus leader RNA was detected in infected BHK-21 cell extracts by hybridization to end-labeled genomic RNA. Similar to the leader RNA of vesicular stomatitis virus, the leader RNA of rabies virus was also found to be associated with the La protein by specific immunoprecipitation with antisera from lupus patients. The 3′ end of the genomic RNA of rabies virus was sequenced, and the size and termination site of leader RNA were determined. In addition, extension of the sequence into the nucleocapsid gene of rabies virus showed an open reading frame for at least 37 amino acid residues. Sequence relationships between rabies virus and vesicular stomatitis virus leader genes and the possible involvement of the La protein in rhabdovirus biology are discussed.

Rabies virus, a rhabdovirus of the *Lyssavirus* genus, is a negative-strand virus containing a nonsegmented RNA molecule. Like vesicular stomatitis virus (VSV), the model system for rhabdoviruses, rabies virus has a bullet-shaped morphology. The RNA is found to be associated with the nucleoprotein (N), forming the nucleocapsid core. The M1 protein as well as the transcriptase activity, presumably supplied by the L protein, are also associated with the nucleocapsid (9, 18). M2 is associated with the lipid envelope, and a glycoprotein is found on the outer surface of the virion. Comparison of the genomic organization (1, 2, 12) as well as patterns of RNA and protein synthesis during the infectious cycle (8, 16, 25) indicate that rabies virus is analogous to VSV. Therefore, its strategy of replication is thought to be similar to that of VSV.

Several differences between the two viruses are noteworthy. Whereas VSV is capable of a productive infection in enucleated cells, rabies virus appears to require nuclear functions (13, 41). In addition, VSV infection is rapid and highly efficient, with progeny virions emerging as early as 4 h postinfection (36). The infectious cycle of rabies virus, however, is very slow, with viral protein synthesis not observed until 6 h postinfection, and progeny virus is released after 24 h postinfection (17). More striking is that, unlike rabies virus, VSV rapidly inhibits host cell macromolecular synthesis, including protein, RNA, and DNA (30, 38, 40). In addition, VSV has cell-killing ability, with most of the infected cells being killed by 12 h postinfection (26, 27, 39). Rabies virus has little or no apparent effect on host macromolecular synthesis and continues to produce progeny virions for several days postinfection (17, 28).

The leader RNA of VSV was originally described as an in vitro transcription product from the exact 3′ end of the genome (6, 7). Leader RNA was subsequently shown to be produced in vivo during the infectious cycle (24). Leader RNA has been proposed by Wagner and co-workers to be the mediator of host cellular RNA synthesis inhibition. This is based on several lines of evidence, including UV inactivation kinetics (37), the requirement for viral transcription (39, 43), and in vitro inhibition of mammalian polymerases II and III by leader RNA in the Manley system (29). Kolakofsky and co-workers have also postulated that the leader RNA contains an encapsidation signal and is used as a decision point for a switch from transcription to replication (4, 5). The leader RNA gene of VSV has also been shown to be the site of viral polymerase entry and initiation (10) as well as a possible site of interaction of the transcription factor, NS protein (21).

We have previously shown that the leader RNA of VSV is associated with the host protein La (22), which is normally found to be associated with RNA polymerase III precursor transcripts (34). To more precisely determine the function of the La protein-leader RNA interaction and to delineate possible viral regulatory regions as well as possible regions mediating host cytopathology, we sequenced the 3′ end of the rabies virus genome. In addition, we detected the leader RNA of rabies virus in infected cells and demonstrated an association between the leader RNA of rabies virus and the host cellular La protein. The possible functions of this complex are discussed, and comparisons are made between the genomes of rabies virus and VSV.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney (BHK-21) cells were maintained as monolayer cultures and used throughout this study. The challenge virus strain of rabies virus was grown, cloned, and stored as previously described (8). For assay of intracellular RNA, cells were harvested at the times indicated below.

Preparation of RNA and cell extract and immunoprecipitation. Infected cells were scraped off the monolayer, washed, and lysed with 1% Nonidet P-40. The nuclei were removed by centrifugation at 2,000 × *g* for 3 min. For total infected cell RNA, the extract was adjusted to 0.1% sodium dodecyl sulfate and phenol extracted. Immunoprecipitations were carried out as previously described (22).

Labeling of genomic RNA and hybridization. Rabies virus genomic RNA was isolated and labeled at its 3′ end with [^{32}P]Cp and T4 RNA ligase as described for VSV genomic RNA (20). Hybridization and nuclease conditions were as previously described (23).

Sequence analysis. Two-dimensional oligonucleotide fingerprints (20) and RNA chemical sequencing (19, 31) were performed as described previously. RNA ladders were pro-

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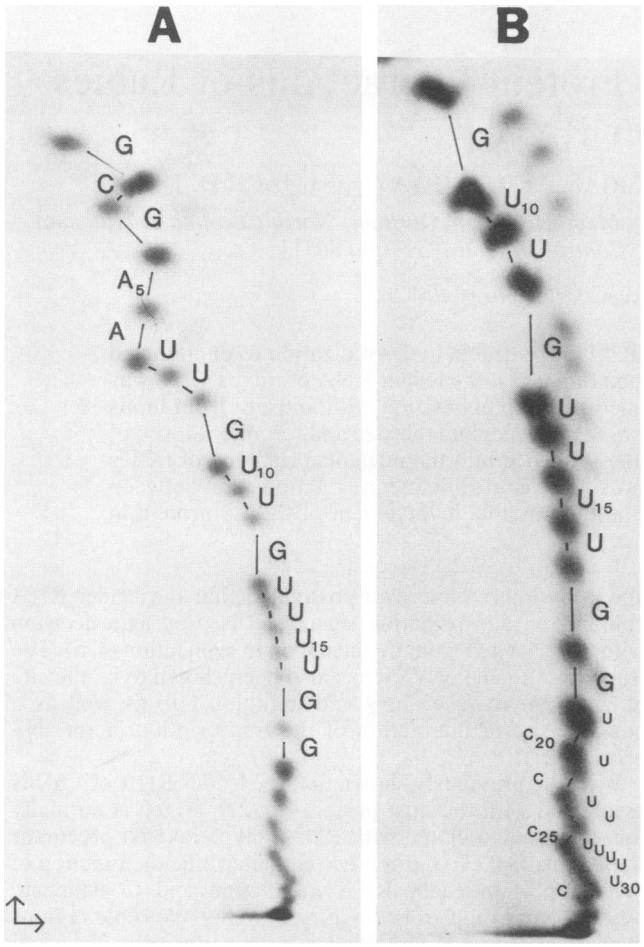

FIG. 1. Two-dimensional oligonucleotide fingerprint analysis of partial alkali digestion products of the 3′-labeled rabies virus genomic RNA. The first dimension (horizontal) is high-voltage electrophoresis from left to right. The second dimension (vertical) is homochromatography from bottom to top. Panels A and B represent the same homomixture for the second dimension but different running times.

duced by alkaline hydrolysis (20). Dideoxy sequencing reactions were carried out as described previously (35).

Acrylamide gel electrophoresis. RNA duplexes and sequence reactions were analyzed on 5, 6, 8, 12, and 20% acrylamide gels as described previously (19, 23).

RESULTS

Sequence determination of the 3′ end of the rabies virus genome. Purified rabies virions were lysed with 1% Nonidet-P-40–0.1% sodium dodecyl sulfate and phenol extracted. The RNA was purified over sodium dodecyl sulfate-sucrose gradients as described above. The 42S RNA was isolated and labeled at its 3′ end with [^{32}P]Cp and T4 RNA ligase. Complete digestion of the end-labeled material with RNase T_2 showed transfer of label to U (data not shown). The end-labeled genomic RNA was subjected to two-dimensional oligonucleotide analysis by the wandering spot method as previously described (20). Panels A and B of Fig. 1 show different running times for the second dimension. The sequence determined by the wandering spot method was used to prepare a complementary DNA oligonucleotide from the first 15 bases for the dideoxy sequencing procedure described below.

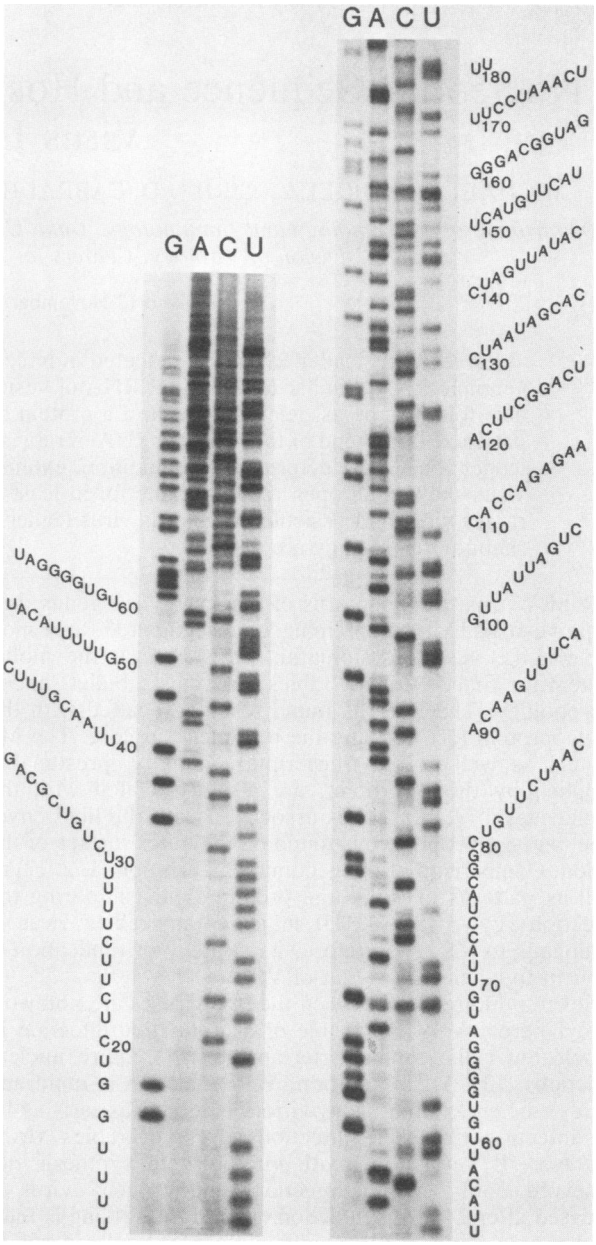

FIG. 2. Chemical RNA sequence analysis of rabies virus genomic RNA. The left panel shows a 12% acrylamide gel and starts at position 13 from the 3′ end. The right panel shows a 5% acrylamide gel and starts at position 54 from the 3′ end.

Figure 2 shows the results of RNA chemical sequencing by the chemical method (19). The sequence shown starts at position 13 and is clearly read through position 180. In other experiments, bases 1 and 13 also were assigned to confirm the data shown in Fig. 1. The RNA sequence shown in Fig. 2 is marked in the minus or genomic sense, whereas the dideoxy sequence shown in Fig. 3 is marked in the plus or message sense. The dideoxy sequencing reactions were performed as described previously (35). A 15-mer DNA primer complementary to the 3′ end of the viral genome was annealed in solution with purified 42S genomic RNA. A 10:1 mixture of deoxynucleoside triphosphates to dideoxynucleoside triphosphates in the presence of [α-^{32}P]dATP was added, and the synthesis of labeled cDNA chains was

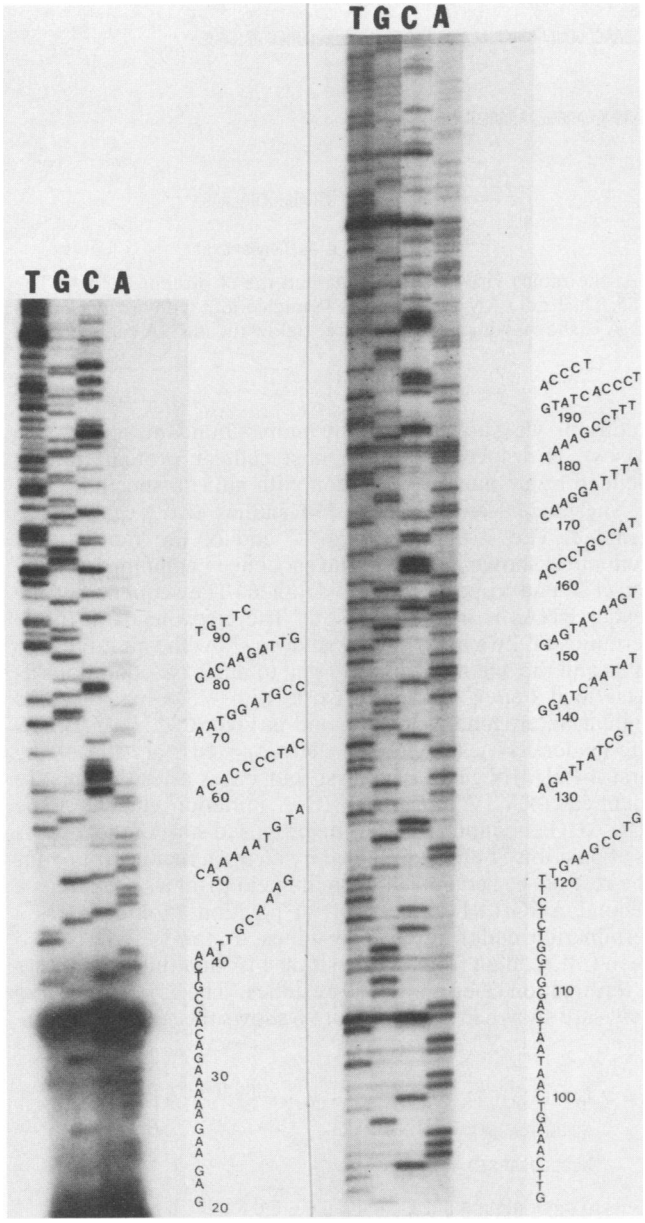

FIG. 3. Dideoxy sequence analysis of the DNA sequence complementary to the 3′ end of the viral genome. The left panel shows an 8% acrylamide gel starting at position 21 from the 3′ end. The right panel shows a 6% acrylamide gel which starts at position 91 from the 3′ end.

directed by reverse transcriptase. The resulting cDNA oligonucleotides were analyzed on acrylamide gels. Positions 21 through 200, complementary to the 3′ end of the genomic RNA, were detectable by this method.

Rabies virus-infected cells containing a leader RNA associated with the La protein. To detect the presence of rabies virus leader RNA, we used a probing protocol similar to the method used to detect the leader RNA of VSV (23, 24). Briefly, an end-labeled genomic probe was annealed in solution with phenol-extracted, infected cell RNA. The resulting duplexes were treated with single-strand-specific ribonucleases (RNases T_1 and A). The protected duplexes were analyzed on acrylamide gels to determine their size. Because a 3′-end-labeled genome was used, only plus-sense

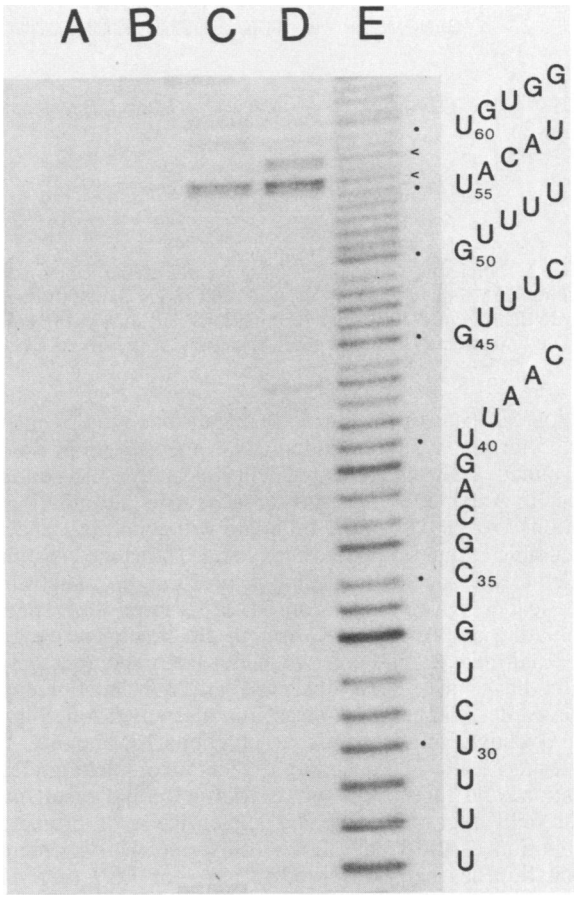

FIG. 4. Detection of rabies virus leader RNA in infected cell extracts and its association with La protein. Lanes A through D were probed for leader RNA with an end-labeled genomic RNA as described in the text. Lanes: A, immunoprecipitation of infected cell extracts with normal human serum; B, total RNA from uninfected BHK-21 cells; C, immunoprecipitation with La-specific antisera; D, total RNA from infected BHK-21 cells. Lane E shows a partial alkali digestion of end-labeled genomic RNA and was indexed by guanidylate spacings as described previously (23). The resulting duplexes were heat denatured before being loaded on a 20% acrylamide gel.

rabies virus transcripts that were contiguous with the 3′ end of the genome would protect the label from nuclease digestion. Thus, only a putative leader RNA transcript and full-length plus-stranded material would protect the labeled probe. To size the resulting duplexes, RNase-resistant species were heat denatured before being loaded onto acrylamide gels and run with a sample of the probe that had been partially digested by alkali to produce a ladder as described above.

Figure 4, lane D shows the results of probing a rabies virus-infected cell extract prepared at 12 h postinfection. Probings were carried out as described above. Figure 4, lane B shows the probing of an equivalent sample of uninfected cell extract. Lane E of Fig. 4 is an alkali ladder which has been indexed to the sequence of the rabies virus genome. Two predominant species were seen in infected cell extracts (Fig. 4, lane D). The two species of rabies virus leader RNA migrated at positions 56 and 58, as indicated by the arrowheads in Fig. 4. The smaller species of leader RNA, terminating at position 56, was present in approximately twofold-greater amounts than the larger species, terminating at

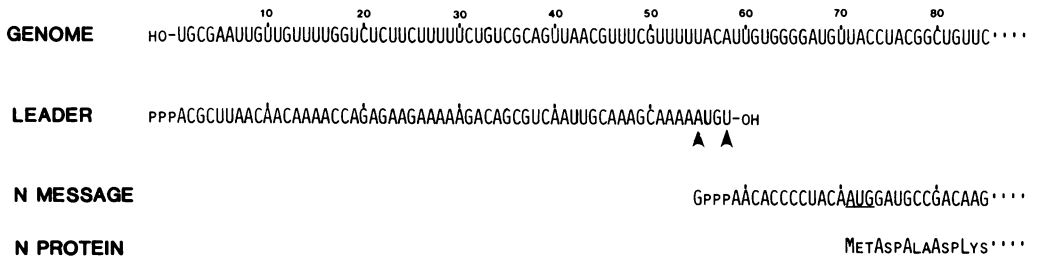

FIG. 5. Nucleotide sequence of the 3′ end of the rabies genomic RNA, the rabies virus leader RNA, and the N protein mRNA. The genomic RNA sequence begins at the 3′ end and is shown through position 85. The leader RNA is shown as 58 nucleotides with arrowheads to indicate the region of imprecise termination. The start of the proposed mRNA is shown with a cap structure. Below the mRNA sequence the first five amino acids are indicated, beginning at the first AUG.

position 58. Due to the nuclease conditions employed, the precise terminus of the leader RNA species could not be determined. RNases T_1 and A will not cleave the genomic probe at A residues. Therefore, a species terminating at positions U_{55} or A_{56} on the template will generate a species of genomic probe 56 nucleotides long. The same reasoning applies to the species migrating at position A_{58}. The rabies virus leader RNAs may consist of a family of species terminating at positions 55 through 58; however, we conclude that there are at least two leader RNA species. Similar results have also been observed at 24 h postinfection; however, less leader RNA was found. In addition, Fig. 4, lane D shows minor bands at positions 43 and 60. The species at position 60 probably reflects incomplete nuclease digestion, and the species at position 43 could result from either mild degradation of the leader RNA or premature termination of the leader RNA transcript. This premature termination has been observed in vitro for VSV (32). This possibility is further supported in that the termination sequence on the template of the 43-nucleotide long species is AC_{44}, with termination occurring at A_{43}, whereas the majority 56-nucleotide long species of leader RNA also terminates with AC_{57} at A_{56}.

To determine whether the leader RNA is associated with the La protein as previously reported for VSV leader RNA (22), immunoprecipitation with sera from patients with systemic lupus erythematosus specific for the La specificity was carried out. As with the total cellular RNA probing described above, infected cells were harvested at 12 h postinfection. After immunoprecipitation and phenol extraction, the RNA was probed as described above. Figure 4, lane C shows the results of probing anti-La-immunoprecipitated infected cell extracts. Figure 4, lane A shows the results with normal human serum. Lane C of Fig. 4 shows one major species of leader RNA migrating at position 56. This species accounts for 87% of the smaller leader RNA species in lane D. Less than 25% of the larger leader RNA species was precipitated by the La antisera. The smaller species of leader RNA (56 nucleotides) represented about 90% of the total La protein-bound leader RNA. Again, a minor species at position 60 was seen and probably represents incomplete nuclease digestion. Figure 4, lane A shows that normal human serum does not precipitate any species of rabies virus leader RNA.

DISCUSSION

We sequenced the 3′ end of the rabies virus genome for ca. 180 nucleotides. By probing infected cell extracts with end-labeled genomic RNA, we detected rabies virus leader RNA as two predominant species of 56 and 58 bases in length. In addition, we showed that the more abundant species (56 bases), is associated with a host cellular protein, La, as defined by immunoprecipitation with anti-La-specific sera.

Nucleotide sequence at the 3′ terminus of the rabies virus genome. The structure of the 3′ end of the rabies virus genome is shown in Fig. 5. The sequence beginning from the exact 3′ end to position 85 is detailed. The sequence of the leader RNAs is indicated below. The arrowheads at the 3′ terminus of the leader RNA sequence show the multiple stop sites and the slight uncertainty due to nuclease conditions as described above. Previous work with VSV has revealed similar heterogeneity in vitro and in vivo (6, 22, 32). Below the leader RNA sequence is the proposed start site of the first mRNA (N gene). The first four bases after the cap are identical to VSV messenger RNA initiation sites (3). The first AUG encountered after the proposed start of the mRNA is at position 71 and is followed by an open reading frame for the rest of the sequence (37 amino acid residues), whereas a second AUG (+1 frame shift) at position 75 encounters a termination codon after 15 residues. A third AUG is also seen (+1 reading frame) at position 147, and this encounters a termination codon after 10 residues. The first five amino acids are shown in Fig. 5. Figure 6 shows the sequence of the

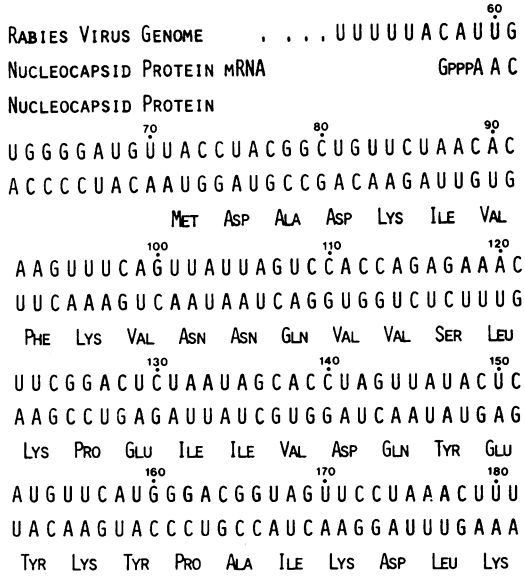

FIG. 6. Sequences of the rabies virus N gene, N mRNA, and N protein. The genomic RNA sequence is shown at the top, with the complementary mRNA sequence in the middle and the amino acid sequence below. The amino acid sequence is marked for 37 residues.

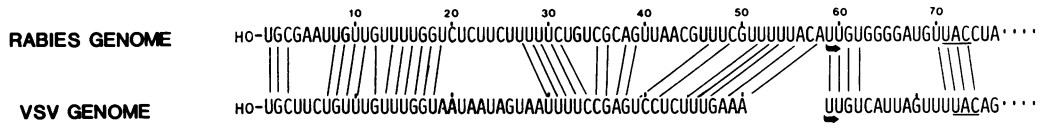

FIG. 7. Comparison of the VSV and rabies virus genomes at the 3′ end. The gap in the VSV genome between the leader gene and N gene was introduced to align the rabies virus and VSV N genes. The start sites of the N gene sequences are shown by arrows. Sequence homologies are indicated by vertical and slanted lines, and the starts of the protein coding sequences are underlined.

proposed 5′ end of the N protein mRNA of rabies virus as well as the partial protein sequence deduced from the nucleotide sequence.

Comparison between the rabies virus genome and VSV. Previous work has demonstrated that rabies virus and VSV have a similar genomic organization for the genes coding for the viral proteins (1, 2, 12). Since the 3′ terminus of the VSV genome is critical for regulatory functions such as polymerase initiation and entry (10, 11, 20), N protein encapsidation, and switching from transcription to replication (4, 5), comparisons between these leader genes should be useful for identifying important sequence relationships.

Figure 7 compares the rabies virus and VSV genomes. The VSV genome has been split to align the regions at the start of the messages (indicated by arrows). Previous work has shown that leader genes of several VSV serotypes range from 50 to 53 bases in length (15). The rabies virus leader gene is 58 bases long. VSV and rabies virus genomes both begin with the same three bases, UGC, whereas among other viruses of the *Vesiculovirus* genus, as many as five bases are conserved at the 3′ end (15). The hexanucleotide sequence UUUGGU that is found invariantly at positions 13 through 18 in the vesiculovirus group is also seen in rabies virus RNA at positions 14 through 19. Kolakofsky and co-workers have proposed that the presence of an A residue in every third position beginning from the 5′ end of the leader RNAs could be part of the encapsidation signal (4, 15). Although found in several vesiculoviruses, this sequence relationship was not found in rabies virus because the leader RNA of rabies virus has a C in the fourth position from the 3′ end. In addition, the lack of U residues in the 5′ portion of the leader RNA of VSV is not found in rabies virus leader RNA in which U_5 and U_6 occur.

At the 5′ end of the leader gene of rabies virus, there is considerable homology with the VSV leader gene. Within 10 bases at the 5′ region, there are 6 bases of direct match and 2 bases with pyrimidine matches. The 3′ end of the RNA transcript is thought to be critical for La protein binding and, therefore, may delineate the efficiency of binding of the La protein. Previous work has indicated that La protein binding may be influenced by sequences as much as 25 bases from the 3′ end of the RNA transcript, however (14, 33, 42). Other vesiculoviruses also have related sequences at the ends of the leader genes. This sequence relationship may reflect signals for transcription termination and reinitiation of the downstream N gene mRNA. Furthermore, these sequences may also be of critical importance for determining the switch from transcription to replication, since this is the first intragenic region on the genome at which termination must be suppressed and complete readthrough allowed to occur. Rabies virus does not contain an obvious intragenic region like that described for VSV (19); however, the A residues at positions 56 and 58 appear to be sufficient. Another common aspect of the leader genes of VSV and rabies virus is the high content (50%) of uridylate residues.

The final base of the leader gene (A_{58}) and the first four bases of the N gene produce a pentanucleotide, AUUGU, which is conserved between rabies virus and VSV. In the case of VSV, all the mRNAs conserve the four bases (AACA) at the start of each mRNA. We presume that by analogy to VSV mRNAs, rabies virus mRNAs also start with a similar tetranucleotide (AACA). The pentanucleotide (AUUGU) could represent a template signal for capping of the nascent transcript. Alternatively, this sequence may be a signal for termination or cleavage of the transcript during transcription (3).

Possible involvement of the La protein in rhabdovirus biology. We detected the leader RNA of rabies virus as two major species, with the smaller species being the more predominant. The smaller species of the leader RNA has been shown to be associated with the cellular protein La. Previous work has implicated the 3′ end of transcripts in La protein binding (14, 33, 42). The 3′ terminal homology between VSV and rabies leader RNAs may reflect the ability of both transcripts to bind the La protein. Also, the exact 3′-terminal bases may reflect different efficiencies of La protein binding between the rabies virus leader RNAs, as has been noted for VSV leader RNAs (22). A model depicting the role of La protein in VSV replication has recently been proposed (J. D. Keene, M. G. Kurilla, J. Wilusz, and J. C. Chambers, *in* D. H. L. Bishop and R. W. Compans (ed.), *Negative Strand Viruses*, in press).

Alternatively, the binding of the La protein to the leader RNA may be fortuitous. A sequence recognized by the polymerase for termination, reinitiation, or capping of a transcript may have enough homology to cellular La protein-binding sites to allow La proteins to bind the leader RNAs. For VSV, the sequence homology between the plus- and minus-strand leader RNAs at their respective 3′ termini is highlighted by the ability of both plus- and minus-strand leader RNAs to bind La protein (22, 42). The specificity of binding is evident because minus-strand leader RNAs missing only eight nucleotides at the 3′ end are not bound to La protein (42).

The rabies virus studies reported here suggest a lack of involvement of the La protein-leader RNA interaction in host cellular RNA synthesis inhibition. The interaction between the leader RNAs of both rabies virus and VSV appear similar in that the majority of the leader RNA species bind La protein. Since the two viruses have such dramatic differences in their effects on host RNA synthesis, it appears that interaction of the leader RNA with the La protein may not be sufficient to inhibit cellular RNA synthesis. However, host shut-off may require a threshold level of leader RNA or the leader RNA-La protein complex, and differences in the amounts of leader RNA synthesized by rabies virus and VSV may account for the inability of rabies virus to inhibit cell RNA synthesis. Also, it is possible that La protein may influence interactions with other host factors that inhibit cellular macromolecular synthesis. The role of the La protein in the replication of rhabdoviruses remains to be elucidated.

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