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In Vitro RNA Transcription by the New Jersey Serotype of Vesicular Stomatitis Virus

II. Characterization of the Leader RNA

RICHARD J. COLONNO AND AMIYA K. BANERJEE*
Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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The New Jersey serotype of vesicular stomatitis virus (VSV) was able to synthesize a small RNA (leader RNA) approximately 70 bases in length similar to the leader RNA synthesized in vitro by the genetically distinct Indiana serotype of VSV. Also, the New Jersey leader RNA contained the same 5'-terminal sequence, ppA-C-G, as the Indiana leader RNA and had a very similar base composition, with 42% AMP, 16% CMP, 18.6% GMP, and 23.4% UMP. The 3'-terminal sequence of the VSV New Jersey genome RNA was determined and found to contain the sequence -Py-G-U_{OH}, again the same as that of the Indiana serotype of VSV. Evidence that the New Jersey leader RNA is transcribed from the 3' end of the genome RNA was obtained from the fact that it can protect the 3'-terminal base of [^{3}H]borohydride-labeled New Jersey genome RNA from RNase digestion. Although the New Jersey and Indiana leader RNAs were similar in many respects, they were unable to form RNase-resistant hybrids when annealed to heterologous genome RNA.

Vesicular stomatitis virus (VSV), a rhabdovirus, contains five structural proteins and a single-stranded genome RNA of negative polarity which sediments at 42S (21). Studies on the Indiana serotype (VSV_{Ind}) have demonstrated that the virion contains an RNA polymerase which sequentially transcribes the genome RNA into a leader RNA followed by five monocistronic mRNA's (1, 2). In the preceding communication (16), it was shown that the serologically distinct New Jersey serotype of VSV (VSV_{NJ}) can also synthesize five mRNA species in vitro which contain very little, if any, sequence homology with the corresponding mRNA species synthesized by VSV_{Ind}. Surprisingly, it was found that a common 5'-terminal sequence, G(5')ppp(5')A-A-C-A-G, identical to that of VSV_{Ind} mRNA's, was present in VSV_{NJ} mRNA's. This observation led us to speculate that the conservation of this sequence was important during evolution of various rhabdoviruses, since this sequence may be an integral part of the recognition site used by the putative processing enzyme during the synthesis of viral mRNA.

In this paper, we further analyze the transcription products of VSV_{NJ} and demonstrate that the serologically distinct New Jersey serotype of VSV is also able to synthesize a leader RNA identical in size to that synthesized by the Indiana serotype. We report that, like VSV_{Ind}, the New Jersey leader has the same ppA-C-G terminus, similar base composition, and is also transcribed from the 3' end of genome RNA, but its base sequence is specific for the VSV_{NJ} genome RNA.

MATERIALS AND METHODS

Purification of VSV. VSV_{Ind} and VSV_{NJ} were grown in baby hamster kidney cells (BHK-21, clone 13), adapted to suspension culture, and purified as described previously (5).

Synthesis and purification of RNA in vitro. Standard in vitro RNA polymerase reactions (1 ml) contained 50 mM Tris-hydrochloride (pH 8.0), 0.1 M NaCl, 5 mM $MgCl_2$, 4 mM dithiothreitol, 0.05% Triton N101, 1 mM ATP, CTP, and GTP, and 0.1 mM UTP. In vitro labeling conditions were as follows: ATP, 250 μCi of [α-^{32}P]ATP (16.4 Ci/mmol) with or without 500 μCi of [^{3}H]ATP (25.8 Ci/mmol) at a final ATP concentration of 0.4 mM; CTP, 250 μCi of [α-^{32}P]CTP (37.4 Ci/mmol) with or without 500 μCi of [^{3}H]CTP (23.1 Ci/mmol) at a final CTP concentration of 50 μM; GTP, 250 μCi of [α-^{32}P]GTP (19.5 Ci/mmol) with or without 500 μCi of [^{3}H]GTP (10.8 Ci/mmol) at a final GTP concentration of 50 μM; UTP, 250 μCi of [α-^{32}P]UTP (17.7 Ci/mmol) with or without 500 μCi of [^{3}H]UTP (35 Ci/mmol) at a final UTP concentration of 50 μM.

Reaction mixtures were incubated with 0.1 mg of purified VSV per ml at 30°C for 5 h. The reactions were terminated by the addition of sodium dodecyl sulfate to 0.5%, and the product RNA was extracted twice with phenol. RNA in the aqueous layers was then purified directly by Sephadex G-50 column

chromatography and oligodeoxyribosylthymine [oligo(dT)]-cellulose chromatography as previously described (15, 16). Product RNA not binding to oligo(dT)-cellulose was precipitated with ethanol and subjected to electrophoresis on 20% polyacrylamide slab gels as previously described (12, 13).

Characterization of the leader RNA. Methodology used in the recovery of leader RNA from polyacrylamide gels, enzymatic digestion, paper electrophoresis, DEAE-cellulose chromatography, and RNA-RNA annealing conditions has been detailed elsewhere (12, 13).

Purification of [^{3}H]borohydride-labeled genome RNA. Fifty micrograms of purified VSV_{NJ} genome RNA was oxidized, followed by reduction with 20 mCi [^{3}H]borohydride, and repurified as previously described (7).

RESULTS

Synthesis of leader RNA. Previous studies using VSV_{Ind} have demonstrated that purified virions are able to synthesize a small leader RNA which is the first RNA product to be synthesized in vitro and is complementary in base sequence to the 3′-terminal end of the 42S genome RNA (12, 13). To determine whether VSV_{NJ} was also capable of synthesizing a leader RNA, in vitro polymerase reactions containing [α-^{32}P]CTP as the labeled precursor were incubated with both VSV_{Ind} and VSV_{NJ}. Polyadenylic acid [poly(A)]-containing mRNA's were removed by oligo(dT)-cellulose chromatography, and the remaining product RNA was analyzed on a 20% polyacrylamide slab gel containing 8 M urea. An autoradiogram of the slab gel (Fig. 1) showed that VSV_{NJ} was capable of synthesizing a small species of RNA (slot II) which migrates identically to the VSV_{Ind} leader RNA shown in slot I.

To determine whether the small RNA species synthesized by VSV_{NJ} was indeed leader RNA, the in vitro polymerase reactions containing [^{3}H]/[α-^{32}P]ATP-, CTP-, GTP-, and UTP-labeled precursors were incubated with purified VSV_{NJ}, and the small RNA species was purified as described in Materials and Methods. Following recovery from gels, each RNA sample was digested with RNAse T_2 and analyzed by paper electrophoresis. Previous studies on VSV_{Ind} leader RNA demonstrated that the 5′-terminal ppAp migrates faster than the ATP marker during paper electrophoresis (12). As shown in Fig. 2, only the ^{3}H radioactive label obtained from ATP (Fig. 2A) was found to migrate in the same position as the ppAp termini of VSV_{Ind} leader RNA. Since ^{32}P was incorporated into the termini from ATP (Fig. 2A) and CTP (Fig. 2B), the 5′-terminal sequence must be ppApC, which is identical to that found with VSV_{Ind} leader RNA. The base composition of the RNA was then

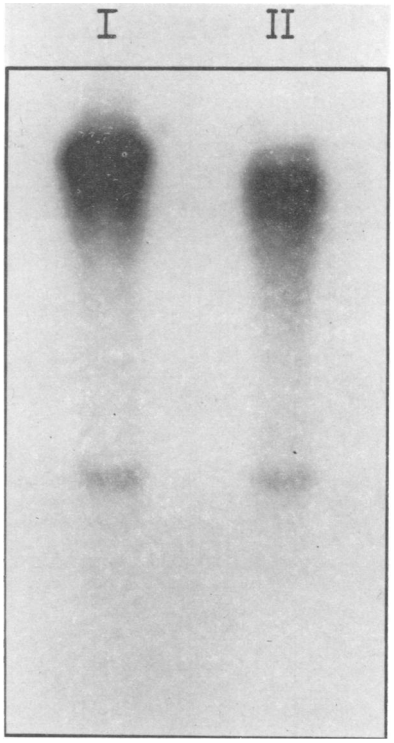

FIG. 1. *Polyacrylamide gel electrophoresis of leader RNA. Product RNAs synthesized in vitro in the presence of [α-^{32}P]CTP by both VSV_{Ind} (I) VSV_{NJ} (II) were purified by phenol-sodium dodecyl sulfate extraction and Sephadex G-50 chromatography. Product RNA was then fractionated by oligo(dT)-cellulose chromatography into poly(A)-containing and non-poly(A)-containing RNA. Product RNA, which did not bind to oligo(dT)-cellulose, was loaded on a 20% polyacrylamide slab gel and autoradiogrammed after electrophoresis for 16 h at 140 V as previously described (12).*

calculated from the quantitative distribution of each label in the four resulting 3′-monophosphates using the method of Josse et al. (17). Results (Table 1) show that both VSV_{NJ} and VSV_{Ind} leaders are very similar; both are rich in AMP, 42% and 47.6%, respectively, and contain fairly similar percentages of CMP, GMP, and UMP.

5′-Terminal analysis of the leader RNA. To determine whether the leader RNA synthesized by VSV_{NJ} contained the 5′-terminal sequence ppA-C-G, [α-^{32}P]CMP-labeled New Jersey leader RNA was purified and digested with RNase T_1. The resulting digest was analyzed by DEAE-cellulose chromatography. Figure 3 shows the RNase T_1 profile obtained following elution with a 0.05 to 0.3 M salt gradient. One would predict, based on previous studies, that

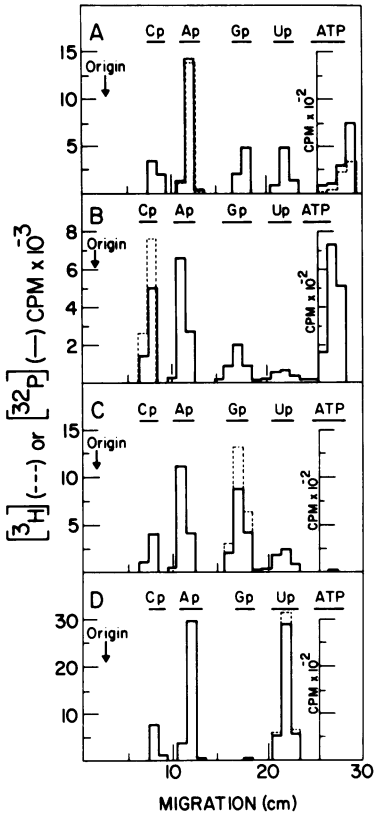

FIG. 2. *Paper electrophoretic analysis of leader RNA following RNase T_2 digestion. Purified leader RNA synthesized by VSV_{NJ} in the presence of (A) [^{3}H]/[α-^{32}P]ATP, (B) [^{3}H]/[α-^{32}P]CTP, (C) [^{3}H]/[α^{32}P]GTP, or (D) [^{3}H]/[α-^{32}P]UTP was purified as described in Fig. 1. Following elution from gels, the leader RNAs were digested with 5 units of RNase T_2 in 0.1 ml of 10 mM Na acetate, pH 4.5, for 5 h at 37°C and then spotted on Whatman 3MM paper. High-voltage paper electrophoresis was at 2,600 V for 60 min in pyridine-acetate buffer (pH 3.5). Marker compounds were included in the samples and located by UV light. The paper was cut into 1-cm strips, and the ^{32}P (——) and ^3H (- - -) radioactivity was determined by scintillation counting.*

the fragment ppA-C-G would elute with a −6.5 charge. The peak eluting at −6.5 was dialyzed and subjected to RNase T_2 digestion followed by paper electrophoresis. Results (Fig. 4) show that the peak of RNA eluting at −6.5 charge yielded predominantly ppAp, which is expected from a pC transfer to ppAp. The small amount of radioactivity which migrated with CMP is due to cross contamination by the peak which elutes at −6 (data not shown). Thus, the 5′-terminal sequence of both the VSV_{Ind} and VSV_{NJ} leader RNAs is ppA-C-G.

3′-Terminal sequence of VSV_{NJ} genome RNA. To determine whether the leader RNA is coded by the 3′-terminal region of the VSV_{NJ} genome RNA, it was important to determine the 3′-terminal sequence of the latter and compare it with the 5′-terminal sequence of the leader RNA. The genome RNA of VSV_{NJ} was isolated from purified virions and labeled with [^{3}H]-borohydride as previously described (7). The [^{3}H]borohydride-labeled genome RNA was divided into three parts. The first was digested with RNase T_2 and analyzed by descending chromatography (Fig. 5A). The radioactivity which migrates from the origin migrates with the marker trialcohol derived from U_{OH}, showing that the 3′-terminal base is U_{OH}. The radioactivity remaining at the origin represents decomposition products frequently encountered with borohydride labeling (13).

A second portion was digested with RNase T_1

TABLE 1. *Base composition of leader RNA*

Base	mol %		Estimated no. of residues[a]	
	Indiana[b]	New Jersey	Indiana	New Jersey
A	47.6	42.0	32	30
C	20.4	16.0	15	12
G	11.1	18.6	8	13
U	20.8	23.5	15	17

[a] The number of base residues was calculated from the base composition and chain length.
[b] Previously determined (12).

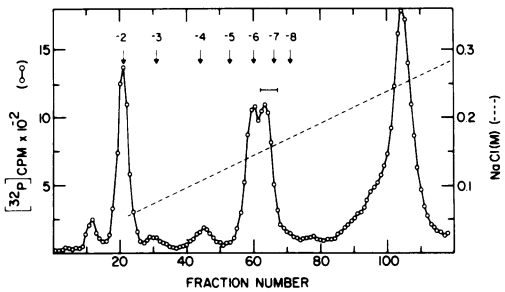

FIG. 3. *DEAE-cellulose chromatography of [^{32}P]-CMP-labeled New Jersey leader RNA following RNase T_1 digestion. Purified [^{32}P]CMP-labeled New Jersey leader RNA was resuspended in 0.1 ml 10 mM Tris-hydrochloride, 1 mM EDTA, 0.3 M NaCl (pH 7.5), and digested with 5 units of RNase T_1 for 1 h at 37°C. The digest was then chromatographed on a column of DEAE-cellulose (0.6 × 17 cm) using a 160-ml salt gradient from 0.05 to 0.3 M NaCl as previously described (12). The NaCl concentration (- - -) was determined from a standard curve based on the conductivity of every fifth fraction. Fractions (1.5 ml) were assayed for ^{32}P radioactivity (○) by Cherenkov counting. Positions of the oligonucleotide markers are indicated by the arrows and are expressed as their net negative charges.*

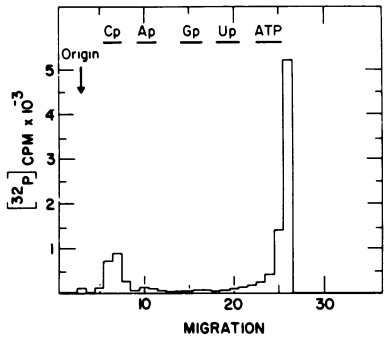

FIG. 4. *Paper electrophoretic analyses of −6.5 oligonucleotide fragment after RNase T_2 digestion. The oligonucleotide obtained after RNase T_1 digestion of ^{32}P-labeled leader RNA which eluted from DEAE-cellulose chromatography at a −6.5 charge (fractions 62 to 67) was desalted by dialysis. Following lyophilization, the oligonucleotide was resuspended in 0.1 ml of Na acetate (pH 4.5) and digested with 5 units of RNase T_2 for 1 h at 37°C. The digest was then analyzed by paper electrophoresis as described in Fig. 2.*

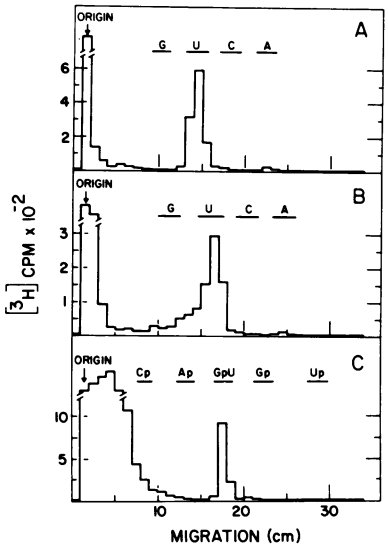

FIG. 5. *3′-Terminal sequence analysis of VSV New Jersey genome RNA. Purified 42S genome (60 μg) was oxidized, followed by reduction with 20 mCi of [^{3}H]borohydride, and repurified as previously described (7). A portion of the [^{3}H]borohydride-labeled genome RNA was divided into three portions. The first two were either digested in 0.1 ml of 10 mM Na acetate, pH 4.5, with 1 unit of RNase T_2 for 5 h at 37°C (A) or digested with RNase T_1 (B) as described in Fig. 3 and analyzed by descending paper chromatography for 16 h in n-butanol–isobutyric acid–H_2O–NH_4OH (30:15:10:1) with the four trialcohol markers as previously described. The third portion was digested in 0.1 ml of 10 mM Tris-hydrochloride, 1 mM EDTA–0.3 M NaCl (pH 7.5) with 10 μg of pancreatic RNase (C) for 1 h at 37°C and analyzed by paper electrophoresis as described in Fig. 2.*

and again analyzed by descending paper chromatography (Fig. 5B). Similarly, the radioactivity which did migrate migrated in the position of uridine trialcohol, indicating that the 5′-proximal base is a G.

The third portion was digested with pancreatic RNase and analyzed by paper electrophoresis (Fig. 6C). The ^{3}H radioactivity migrated with the marker GpU, demonstrating that the third base from the 3′ terminus is a pyrimidine. These results are again identical to those found with the genome RNA from the Indiana serotype and show that the 3′ terminus of the VSV_{NJ} genome RNA is -Py-G-U_{OH}.

Mapping of leader RNA at 3′ terminus of genome RNA. Previous studies on the VSV_{Ind} leader RNA demonstrated that it is transcribed from the 3′ end of Indiana genome by the virion polymerase (13). To illustrate that this is also the case with the New Jersey serotype, ^{32}P-labeled VSV_{NJ} leader RNA was hybridized to purified [^{3}H]borohydride-labeled genome RNA, and the resulting RNA duplex was treated with RNase T_2. The RNase-resistant material was then analyzed by electrophoresis on a 20% polyacrylamide gel (Fig. 5). The ^{3}H-labeled terminus of the genome was protected from RNase degradation by leader RNA, as evidenced by the fact that both ^{3}H and ^{32}P radioactive labels comigrated into the gel. Again, the ^{3}H radioactivity remaining at the top of the gel represents a decomposition product previously observed with this type of label (13). In control experiments, when [^{3}H]borohydride-labeled genome RNA was hybridized to 14.5S mRNA (N-mRNA) or self-annealed (13), the ^{3}H-labeled terminus was not protected from RNase digestion (data not shown). These results indicate that the leader RNA synthesized by the VSV_{NJ} and VSV_{Ind} are both transcribed from the 3′ end of the genome RNA.

Leader RNAs do not share base sequence homology. To examine the extent of base sequence homology between the two leader RNAs, [^{32}P]UMP-labeled leader RNA was synthesized using both serotypes, purified, and used in hybridizations either with its homologous genome RNA from which it was transcribed or with the serologically distinct genome RNA. The hybridization conditions were identical in all cases. Following hybridization, the RNA-RNA duplexes were ethanol precipitated and digested with RNase T_2 to degrade any single-stranded RNA. The digests were loaded directly onto a 20% polyacrylamide slab gel and subjected to electrophoresis. An autoradiogram of the gel

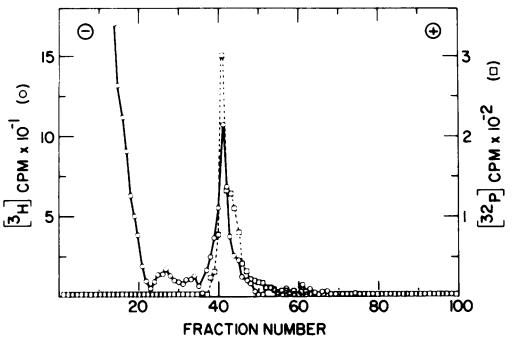

FIG. 6. *Protection of [^{3}H]borohydride-labeled genome RNA by leader RNA. Purified 42S genome RNA was labeled specifically at the 5′ terminus with ^{3}H-labeled borohydride and repurified as previously described (7). Annealing was performed in 0.1 ml of 0.03 M Na citrate–0.3 M NaCl (pH 7.0) with 3 μg of [^{3}H]borohydride-labeled genome RNA and 60 ng of [^{32}P]CMP-labeled leader RNA for 45 min at 72°C and slow cooled to 40°C. The RNA duplex was ethanol precipitated, resuspended in 0.05 ml of 20 mM Na acetate–0.3 M NaCl (pH 4.5) and digested with 1.5 units of RNase T_2 for 30 min at 37°C. The digest was then analyzed by electrophoresis on a 20% polyacrylamide disk gel for 6 h at 5 mA as previously described (13). The gel was sliced into 1-mm slices by a Gilson automatic gel crusher, and the fractions were incubated at 50°C in 0.6 ml of 15% H_2O_2 for 16 h and then assayed for ^{3}H (○) and ^{32}P (□) radioactivity in 7.5 ml of Aquasol 2.*

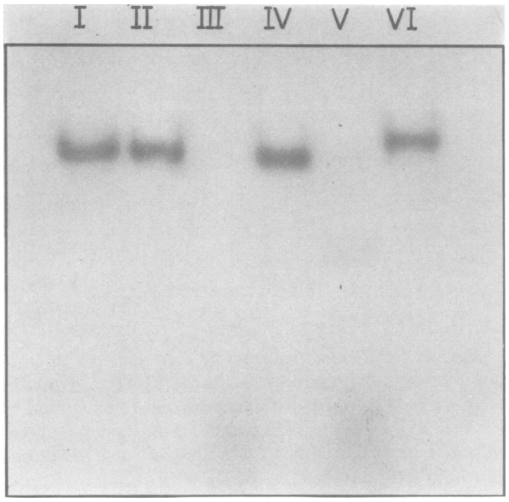

FIG. 7. *Cross-hybridization of leader RNAs to Indiana and New Jersey genome RNAs. [^{32}P]CMP-labeled leader RNAs synthesized by both VSV_{Ind} and VSV_{NJ} were purified as described in Materials and Methods and used in annealing experiments with unlabeled 42S genome RNA from both serotypes. Annealing conditions were as follows: 2 ng of ^{32}P-labeled leader RNA was incubated with 1 μg of 42S genome RNA in 0.1 ml of 0.03 M Na citrate–0.3 M NaCl (pH 7.0) for 45 min at 72°C, slowly cooled to 40°C, and ethanol precipitated. The RNA duplexes were digested with RNase T_2 as described in the legend to Fig. 2, analyzed by electrophoresis on a 20% slab gel for 2.5 h at 100 V, and autoradiogrammed. Slots I and IV are Indiana and New Jersey leader RNAs, respectively, loaded directly into the gel as controls. Slots II and VI are Indiana and New Jersey leader RNAs after annealing to their respective genome RNA and RNase digestion. Slots III and V are Indiana and New Jersey leader RNAs annealed to New Jersey and Indiana genome RNAs and RNase digestion.*

(Fig. 7) shows that unannealed VSV_{Ind} leader RNA (I) and VSV_{Ind} leader RNA annealed to its genome RNA (II) migrated at nearly identical rates, whereas unannealed VSV_{NJ} leader RNA (IV) moved differently than VSV_{NJ} leader RNA annealed to its genome RNA (V). Cross annealing of VSV_{Ind} leader RNA to VSV_{NJ} genome RNA (III) and vice versa (VI) showed virtually no RNase-resistant radioactivity. Instead, the digested leader RNAs can be seen at the bottom of the gel migrating as presumptive mononucleotides. Although the leader RNAs of both the serotypes have the same size (Fig. 1), 5′-terminal sequence ppA-C-G, and similar base compositions, there is sufficient difference in their base sequences that stable RNase-resistant duplexes cannot be formed by hybridization with the heterologous genome RNA.

DISCUSSION

In this communication we have demonstrated that the virion-associated RNA polymerase of VSV_{NJ} is able to synthesize a small RNA species which is identical in many respects to the leader RNA species synthesized by VSV_{Ind}. Both leader RNA species contain an identical 5′-terminal sequence, ppA-C-G, are rich in adenosine content (average, 45%), and are approximately 70 bases long. The 5′-terminal sequence of the VSV_{NJ} leader RNA appears to be complementary to the 3′-terminal sequence of the genome RNA, since the latter sequence was found to be -Py-G-U_{OH} (Fig. 5). Moreover, by hybridization of the leader RNA with [^{3}H]borohydride-labeled VSV_{NJ} genome RNA, it was found that the 3′-terminal base of the genome RNA can be protected from RNase digestion (Fig. 6), indicating that the leader RNA is complementary to the 3′-terminal region of the genome RNA. In this respect, the VSV_{NJ} leader RNA is similar to the VSV_{Ind} leader RNA. Despite these similarities, the leader RNA species of VSV_{NJ} and VSV_{Ind} do not share appreciable sequence homologies (Fig. 7). Thus, the leader RNA and the mRNA products synthesized in vitro by VSV_{NJ} and

VSV_{Ind} are similar in number (16), but quite distinct as regard to their base sequence, although each contains the common 5'-terminal sequence G(5')ppp(5')A-A-C-A-G in the mRNA's and ppA-C-G in the leader RNA.

Chang et al. (10) have studied the transcription products of the five serologically distinct rhabdovirus isolates, VSV_{Ind}, VSV_{NJ}, Cocal, Chandipura, and Piry. Although the RNA products were not characterized with respect to size, all the RNA products were found to contain a common 5'-terminal sequence, pppA-C-G, when [γ-^{32}P]ATP was used as the labeled precursor. Since previous studies indicate that all the mRNA species have capped 5' termini (2), the RNA species containing pppA-C-G may represent leader RNA molecules. We have shown that at least in VSV_{Ind} and VSV_{NJ}, this sequence is present only in the leader RNAs. The above two observations, taken together, indicate that synthesis of leader RNA may be a common phenomenon in all rhabdoviruses, and a common 5'-terminal (p)ppA-C-G may be present in all leader RNAs. In addition, recent studies on the paramyxovirus Newcastle disease virus have demonstrated that it also synthesizes a unique leader RNA in vitro which appears identical in size to the VSV leader RNA and contains 5'-terminal ppG-C and ppA-C sequences (4). Thus, it appears that the synthesis of leader RNA may be a general strategy for the initiation of RNA synthesis by the negative strand viruses with unsegmented genome RNAs.

The results presented in this paper and in the preceding one provide an insight into the possible mechanisms of transcription and replication of rhabdoviruses. As shown in the preceding paper and by others (16, 18, 20), the mRNA's of VSV_{NJ} and VSV_{Ind} do not share appreciable sequence homologies. Nevertheless, both the serotypes have a cross-reacting group-specific antigen in the ribonucleoprotein cores (9). This apparent contradiction has not yet been resolved. Our data clearly indicate that at least two vital nucleotide sequences have been conserved in the RNA products synthesized by the two serotypes of VSV: the 5'-terminal ppA-C-G sequence of the leader RNAs and the 5'-terminal G(5')ppp(5')A-A-C-A-G sequence of the mRNA's. Likewise, the 3'-terminal sequence ...Py-G-U_{OH} is common to the genome RNAs of both serotypes. The above results strongly suggest that the nucleotide sequence involved in binding and initiation by the virion-associated polymerase may be similar for VSV_{Ind} and VSV_{NJ}. Although three common bases may not be sufficient to impart a recognition signal for binding of the polymerase, the extent of sequence homology at the 3' end of the genome RNAs of both the serotypes is still not known. If indeed both genome RNAs contain an appreciable degree of sequence homology at the 3' ends, the RNA polymerases (L or NS proteins or both) of both the viruses may recognize the same 3' ends of genome RNA and initiate transcription with the synthesis of two different kinds of leader RNA. Further evidence to support this prediction is the fact that of all defective interfering particles of VSV that were assayed for in vitro RNA polymerase activity, only the defective particle whose genome RNA contained sequences which code for the leader RNA was able to synthesize complete mRNA's in vitro (14). The putative processing of the mRNA's may also occur at identical sequences, since the mRNA's of both serotypes of VSV contain a common 5'-terminal sequence that may signal the site of processing. The above prediction can readily interpret the previous findings that (i) VSV_{NJ} can complement the transcription defect of temperature-sensitive G-114, a mutant of VSV_{Ind}, at the nonpermissive temperature (19), and (ii) VSV_{NJ} can support replication of a defective interfering particle of Indiana serotype which lacks the L protein gene (11). Although direct in vitro reconstitution experiments (8) failed to show that VSV_{NJ} L and NS proteins can complement the corresponding proteins of VSV_{Ind}, the VSV_{NJ} virus used had very low initial transcriptase activity (approximately 5% that of VSV_{Ind}). Thus, the reconstitution experiments may not have been carried out under optimum conditions for the transcriptase activity. Further in vitro experiments should show whether VSV_{NJ} L and NS proteins can directly interact with VSV_{Ind} genome RNA-N protein complex.

Finally, the presence of common 5'-terminal sequences of the mRNA's and the leader RNAs of VSV_{NJ} and VSV_{Ind} indicate that these sequences may have been conserved if these isolates have evolved from a common ancestor. Clearly, these sequences are important in relation to initiation of RNA transcription and biosynthesis of virus-specific mRNA's.

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