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Sequential Synthesis of 5′-Proximal Vesicular Stomatitis Virus mRNA Sequences

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We examined the kinetics of synthesis in vitro of the 5′ ends of the vesicular stomatitis virus mRNAs by analysis of specific RNase T_1 oligonucleotides located near the 5′ ends of the mRNAs. Our results indicate that, like synthesis of full-length mRNAs, the 5′ ends of the mRNAs are synthesized sequentially, following the gene order N, NS, and M. Additional experiments with UV-irradiated virus demonstrated that synthesis of the mRNA regions containing these oligonucleotides is dependent on synthesis of the mRNA from the preceding gene. These results are inconsistent with a model of vesicular stomatitis virus transcription involving simultaneous initiation and presynthesis of leader RNAs 30 to 70 nucleotides long for each mRNA. We also characterized two small RNA species whose synthesis is highly resistant to UV irradiation. Partial sequence analysis indicates that these RNAs are a 5′-capped fragment of the N mRNA and a 5′ fragment of the leader RNA.

The RNA genome of vesicular stomatitis virus (VSV) is transcribed in vitro by a virion-associated RNA polymerase into six discrete RNA species (3). These include the 47-nucleotide leader RNA (6) and the five capped and polyadenylated mRNAs encoding the viral proteins N, NS, M, G, and L (3, 5, 10, 13). Experiments measuring the effect of UV irradiation on viral gene expression indicated that the virion-associated RNA polymerase initiates transcription at a single site located at the 3′ end of the genome RNA and transcribes the genes sequentially in the order 3′-leader-N-NS-M-G-L-5′ (1, 2). Two mechanisms were originally proposed to account for sequential transcription (1–3). In the cleavage model, the monocistronic mRNAs are generated via cleavage and processing of a precursor RNA. In the stop/start model, the mRNAs are generated by termination and reinitiation of transcription at each intercistronic region.

A third model, which we will call the simultaneous initiation model, has recently been proposed (24). This model is based on the observation that, in addition to the leader RNA, at least three discrete, low-molecular-weight RNA species could be detected almost immediately after activation of the virion polymerase in vitro. A 5′-terminal sequence of two of these small RNAs is in agreement with the sequence at the 5′ ends of the N and NS mRNAs except that the RNAs had 5′-triphosphate termini instead of 5′ cap structures (24). The third RNA was tentatively identified as being derived from the 5′ end of the M mRNA. From these results it was suggested that initiation of transcription occurs simultaneously at the beginning of each gene, resulting in the transcription of 5′ leader RNAs approximately 30 to 70 nucleotides in length from the 3′ end of each gene. These leader RNAs are subsequently elongated into full-length mRNAs, but only after transcription of the preceding gene is complete. This model accounts equally well for the fact that overall mRNA synthesis is sequential.

Both the cleavage and the stop/start models predict that the 5′-proximal region of an mRNA will not be synthesized until transcription of the preceding gene is complete. In contrast, if the simultaneous initiation model is correct, the 5′-proximal mRNA region of each mRNA will be synthesized at the same time, immediately after activation of the virion polymerase in vitro. In an earlier report we demonstrated sequential transcription of full-length VSV mRNAs by hybridization of mRNA synthesized in vitro to cDNA clones of the VSV mRNAs (9). Using this technique, we were unable to determine the kinetics of synthesis of the 5′ ends of the VSV mRNAs because the cDNA clones do not contain those sequences corresponding to the 5′-terminal regions of the VSV mRNAs.

To determine if significant amounts of 5′-proximal mRNA synthesis occur shortly after polymerase activation, we have made use of the fact that the sequences of the leader RNA (6)

and the N, NS, M, and G mRNAs (8, 20) are now known. Knowledge of these sequences allows us to predict the size and sequence of every RNase T_1 oligonucleotide derived from the leader RNA or the N, NS, M, and G mRNAs. This information has facilitated identification of three unique oligonucleotides derived from the 5'-proximal regions of the N, NS, and M mRNAs. Analysis of the kinetics of appearance of these 5' oligonucleotides has allowed us to determine the kinetics of synthesis of the 5' ends of the VSV mRNAs. In addition, these 5' oligonucleotides have been useful in determining the effects of UV irradiation on the synthesis of 5'-proximal mRNA sequences.

MATERIALS AND METHODS

Virus growth and in vitro transcription. BHK-21 cells, adapted to suspension culture, were used for virus growth. The details of infection and virus purification were as previously described (21, 22). A cloned VSV stock (San Juan strain of the Indiana serotype) was used for in vitro synthesis of VSV mRNA as described previously (9, 22) except that the ribonucleoside triphosphate concentrations were: ATP, 1 mM; CTP, 0.5 mM; UTP, 25 μM; and [α-^{32}P]GTP (36 Ci/mmol), 27.5 μM for GTP labeling. When UTP was used as the source of label, the GTP concentration was 25 μM and the [α-^{32}P]UTP concentration (36 Ci/mmol) was 27.5 μM. Reaction mixtures were preincubated for 5 min at 30°C without $MgCl_2$, and transcription was initiated by the addition of $MgCl_2$ to a final concentration of 5 mM. Samples taken at various times after initiation were diluted into 0.5% sodium dodecyl sulfate and 0.2 M sodium acetate to stop the reaction. UV irradiation of VSV virions was as previously described (9) except that the virus samples were exposed to the UV source for 0 or 40 or 1.5, 10, or 20 min. Synthesis of [α-^{32}P]GTP-labeled RNA using UV-irradiated virions was allowed to proceed for 2 h after the addition of $MgCl_2$ as described above.

Fractionation and sequence analysis of RNase T_1 oligonucleotides. RNA samples were extracted with phenol and precipitated with ethanol using 5 μg of carrier RNA. The precipitated RNA samples were resuspended in 2 μl of a solution containing 20 U of RNase T_1 per ml of 0.05 M ammonium acetate, pH 5.2, and incubated for 1 min at 90°C. After incubation at 90°C, 3 μl of the RNase T_1 mixture was added to the RNA sample which was then incubated at 37°C for 30 min. The products of digestion with RNase T_1 were subjected to two-dimensional gel electrophoresis as described previously (7, 18) except that the gel dimensions were 1 mm by 10 cm by 40 cm (first-dimension gel) and 1 mm by 25 cm by 30 cm (second-dimension gel). Oligonucleotides identified by autoradiography were excised and eluted in 0.4 ml of water for 2 to 6 h at 37°C. After lyophilization, the RNA samples were resuspended in 4 μl of 10 mM Tris-hydrochloride–1 mM EDTA (pH 7.5) and divided into two samples of equal volume. One of these samples was digested further with RNase A and then analyzed by electrophoresis on DEAE paper (DE 81, Whatman Inc.) at pH 3.5. The second sample was digested with a mixture of RNases A, T_1, and T_2 and analyzed by electrophoresis at pH 3.5 on Whatman 3MM paper (Whatman Inc.). These procedures have been reported in detail elsewhere (4, 17, 18).

Materials. RNases T_1 and T_2 were purchased from Calbiochem, La Jolla, Calif., and RNase A was from Worthington Diagnostics, Freehold, N.J. Bacterial alkaline phosphatase and calf intestine phosphatase were from Boehringer Mannheim Corp., Indianapolis, Ind. [α-^{32}P]GTP and [α-^{32}P]UTP were purchased from Amersham Corp., Chicago, Ill., and unlabeled nucleoside triphosphates were from P-L Biochemicals, Milwaukee, Wis.

RESULTS

Identification of 5' oligonucleotides. To determine if the 5' ends of the VSV mRNAs are initiated simultaneously or sequentially, ideally one would like to examine the time of appearance of those oligonucleotides corresponding to the exact 5' termini of the different mRNAs. This is not possible because all five VSV mRNAs contain the identical 5'-terminal sequence, m^{7}G5'ppp5'AACAG (16, 18, 19). However, examination of the complete sequence of the leader RNA (6) and of the N, NS, M, and G mRNAs (8, 20) revealed four large T_1 oligonucleotides located within the first 50 nucleotides of the 5' ends of these RNAs. These include a 28-mer within the leader RNA, which we will call L3 (because it is the third oligonucleotide from the 5' end of the leader RNA), an 11-mer in the *N* mRNA (N2), a 15-mer in the NS mRNA (NS4), and a 22-mer in the M mRNA (M5). The sequences of these oligonucleotides and their positions within the mRNAs are shown in Fig. 1.

To determine if we could identify these oligonucleotides in total RNase T_1 digests of VSV RNAs, we first synthesized VSV RNA in vitro in the presence of [α-^{32}P]GTP. This choice of label guarantees that every RNase T_1 product, except those arising from the 3' terminus of an RNA molecule, will contain at least one radioactive phosphate. The RNA was extracted with phenol, precipitated with ethanol, and digested to completion with RNase T_1. An autoradiogram of the resulting oligonucleotides separated by two-dimensional gel electrophoresis (7) is shown in Fig. 2. To achieve maximum separation of T_1 oligonucleotides, second-dimension electrophoresis was allowed to proceed for 16 to 18 h at 600 to 800 V. These conditions of electrophoresis result in the loss of most T_1 oligonucleotides less than 10 nucleotides in length. Therefore, only those RNase T_1 oligonucleotides about 10 nucleotides or longer are visible on the autoradiogram. The spots vary in intensity because the different mRNAs are synthesized in different molar amounts (9, 25) and because some of the spots in Fig. 2 are actually mixtures of two or more oligonucleotides of similar lengths. Also,

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Leader RNA      ppACGAAGACAAACAAACCAUUAUUAUCAUUAAAAGGCUCAGGAGAAAA(C)
                      1        10        20        30        40

5' N mRNA     m7GpppAACAGUAAUCAAAAUGUCUGUUACAGUCAAGAGAAUCAUUGACAACACAG
                      1        10        20        30        40

5' NS mRNA    m7GpppAACAGAUAUCAUGGAUAAUCUCACAAAAGUUCGUGAGUAUCUCAAGUCCU
                      1        10        20        30        40

5' M mRNA     m7GpppAACAGAUAUCACGAUCUAAGUGUUAUCCCAAUCCAUUCAUCAUGAGUUCC
                      1        10        20        30        40

5' G mRNA     m7GpppAACAGAGAUCGAUCUGUUUCCUUGACACUAUGAAGUGCCUUUUGUACUUA
                      1        10        20        30        40
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FIG. 1. Sequences of 5′ oligonucleotides and their positions within the leader RNA and the N, NS, M, and G mRNAs. The sequence of the leader RNA (6) and the first 50 nucleotides of the N, NS, M, and G mRNAs (8, 20) are shown with the RNase T_1 oligonucleotides L3, N2, NS4, and M5 shaded.

those oligonucleotides that are followed by a G residue will contain two radioactive phosphates.

A diagram of the autoradiogram is shown in Fig. 2B in which several of the oligonucleotides are numbered. These oligonucleotides and approximately 30 additional oligonucleotides were eluted from this gel or gels of lower complexity (see below) and divided into two samples of equal volume. One of these samples was digested with RNase A, and the resulting products were separated by electrophoresis on DEAE paper at pH 3.5. The second sample was digested using a mixture of RNases A, T_1, and T_2, and the products were separated by paper electrophoresis at pH 3.5. Because the position of an oligonucleotide relative to the dye markers is dependent on its length and the ratio of C+A to U+G (7), these two parameters can be used, as a first approximation, to locate a particular oligonucleotide on the gel. Combined with the sequence analysis described above, this allowed absolute identification of those oligonucleotides listed in Table 1.

The partial sequences deduced from the results of these secondary digestions as well as a similar analysis of the RNase T_1 products of

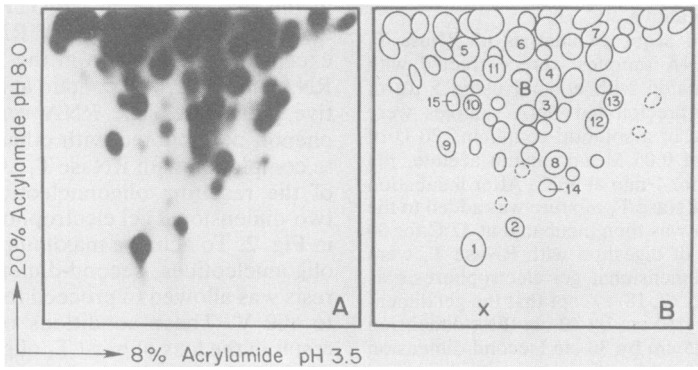

FIG. 2. Two-dimensional gel electrophoresis of the RNase T_1 digestion products from VSV RNA synthesized in vitro. RNase T_1 digestion products of VSV RNA labeled for 2 h with [α-^{32}P]GTP were separated in the first dimension by electrophoresis on an 8% acrylamide gel containing 6 M urea and 25 mM citric acid at pH 3.5. Separation in the second dimension was by electrophoresis on a 20% acrylamide gel containing 40 mM Tris-citrate gel at pH 8.0 (7). The arrows indicate the direction of first- and second-dimension electrophoresis. (A) Autoradiogram of the resulting product RNAs. (B) Diagram of the autoradiogram in (A), where X and B indicate the position of the xylene cyanol and bromophenol blue markers, respectively. The numbers refer to those oligonucleotides listed in Table 1.

TABLE 1. Partial sequence analysis and identification of RNase T_1 oligonucleotides from Fig. 2

Spot[a]	RNA	Position[b]	Sequence[c]
1	Leader	7–34	ACAAACAAACCAUUAUUAUCAUUAAAAG(G)[d] AUU AAAAG(G)
2	N	125–153	AUUCCUCUUUACAUCAAUACUACAAAAAG(U) AU CU UU AAUACU AAAAAG(U)
3	N	57–71	UUCCAAAACUUCCUG(C) UU AAAACU CUG(A or C)
4	N	204–218	UAUCAAUCAUACAUG(U) AU AAU AUG(U)
5	N	187–197	CCUCAAAUCCG(G) CU AAAU CG(G)
	N	289–299	AAUAAACAUCG(G) AAU AUCG(G)
6	N	6–16	UAAUCAAAAUG(U) AAU AAAAUG(U)
7	N	102–111	AUUACUUCAG(A) AU ACUU AG(A or C)
8	N	983–1,001	UAUACAUCUCUUACUACAG(C) AU CU UUACU AG(A or C)
9	N	1,068–1,085	AUAACAAAUACACUCCAG(A) AU AAAU ACU AG(A or C)
10	NS	15–29	AUAAUCUCACAAAAG(U) AUAAUCU AAAAG(U)
	NS	627–641	ACUUUCCAACCCAAG(A) ACUU AAG(A or C)
11	N	626–638	UUCAAAAAACAUG(A) UU AUG(A or C)
12	N	872–887	UCUCCAUAUUCUUCCG(U) CU AU UU CG(U)
13	NS	161–175	CCCUCUUAUUUUCAG(G) CU UUAU AG(G)
14	M	23–44	UUAUCCCAAUCCAUUCAUCAUG(A) UUAU AAU AUG(A or C)
15	M	109–123	CACCACCCCCUUAUG(A) UUAUG(A or C)

[a] The spot number refers to those oligonucleotides identified in Fig. 2.

[b] Position refers to the position in nucleotides from the 5' end of the mRNA.

[c] The upper sequence is from the published sequences of the VSV leader RNA (6) or the N, NS, and M mRNAs (8, 20). The lower sequences were determined by secondary sequence analysis of [α-^{32}P]GTP- and [α-^{32}P]UTP-labeled RNA as described in the text. In most cases, the order of the internal RNase A products could not be determined except by alignment with the known sequence. For example, internal sequences such as AAUACU (spot no. 2) were deduced only after observing radioactively labeled AAU and AC among the RNase A digestion products of [α-^{32}P]UTP-labeled T_1 oligonucleotide no. 2. In addition, because the radioactively labeled species present after RNase A or RNase A, T_1, and T_2 were not excised from the DEAE or Whatman 3MM paper and quantitated, we have not indicated multiple species present in the same oligonucleotide. For example, the sequence AUU appears three times in oligonucleotide no. 1 but is indicated only once.

[d] Parentheses indicate the 3' nearest neighbor of a particular oligonucleotide. If no radioactively labeled GMP residue is present after RNase T_2 digestion of either an [α-^{32}P]GTP- or [α-^{32}P]UTP-labeled T_1 oligonucleotide, this indicates that the 3' nearest neighbor of that particular oligonucleotide must be either A or C.

[α-^{32}P]UTP-labeled RNA are given in Table 1. In every case the partial sequence can be aligned within the complete sequence of the corresponding oligonucleotide listed in Table 1 and is incompatible with the sequence of any other RNase T_1 oligonucleotide of similar size and C+A to U+G ratio derived from the leader RNA or the N, NS, M, or G mRNAs. Although the complete sequence of the L mRNA has not been determined, any oligonucleotides derived from the L mRNA should not interfere with our analysis because the molarity of the L mRNA is low relative to the other mRNAs (25). Many of the spots are mixtures of two or more oligonucleotides in the RNA synthesized for 2 h in the presence of [α-^{32}P]GTP (Fig. 2) but are unique in fingerprints of RNA synthesized in the presence of [α-^{32}P]GTP for shorter periods of time (Fig. 3A). Those oligonucleotides listed in Table 1 which are not unique in Fig. 2 were identified by analysis of the RNase T_1 products of RNA synthesized in shorter reactions.

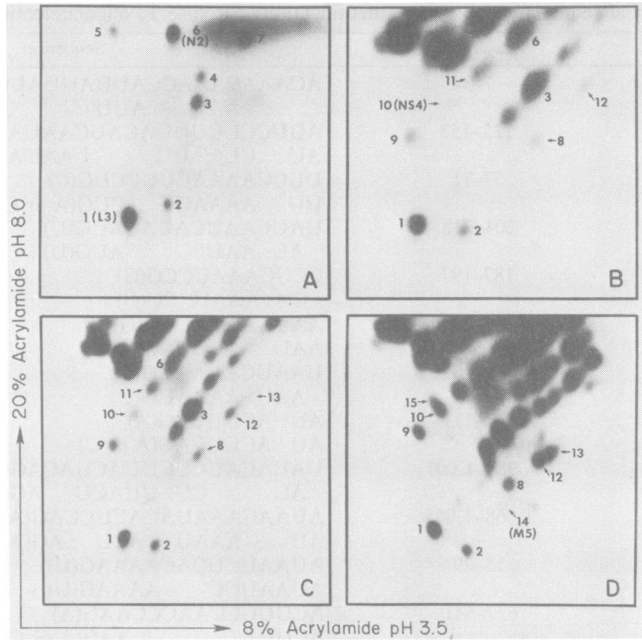

FIG. 3. Analysis of the kinetics of appearance of RNase T_1 oligonucleotides. RNA samples synthesized in the presence of [α-^{32}P]GTP for (A) 5 min, (B) 15 min, (C) 20 min, and (D) 30 min (after initiation of the in vitro transcription reaction) were extracted with phenol, precipitated with ethanol, and digested with RNase T_1. Autoradiograms of the resulting product RNAs separated by two-dimensional gel electrophoresis as described in Fig. 2 and the text are shown. The numbers refer to those oligonucleotides identified in Table 1 and the text. Some of the oligonucleotides (e.g., no. 2) are slightly skewed on some of the gels due to slight variations in the conditions of electrophoresis.

Kinetics of synthesis of the 5′ ends of VSV mRNAs. When it was clear that we could identify the oligonucleotides L3, N2, NS4, and M5, we examined the kinetics of appearance of these oligonucleotides during in vitro transcription. Total VSV RNA synthesized after 5, 15, 20, or 30 min was digested with RNase T_1, and the products were separated by two-dimensional gel electrophoresis. Autoradiograms of the resulting oligonucleotides from each of the four time points are shown in Fig. 3.

All of the oligonucleotides (greater than 10 nucleotides in length) synthesized by 5 min (Fig. 3A) were derived from either the leader RNA (L3) or the 5′ half of the N mRNA (oligonucleotides 2 to 7). We could not detect NS4 or M5 even after exposing the gel for a period of time which would allow detection of an oligonucleotide that is 14 times less abundant than the leader oligonucleotide L3 (data not shown). By 15 min (Fig. 3B), the complexity of the product RNA had increased considerably. Several oligonucleotides derived from the 3′ half of the N mRNA (oligonucleotides 8, 9, 11, and 12) and a faint spot which corresponds to NS4 (oligonucleotide 10) become visible at this time. There is no evidence of M5, which should migrate just below the 3′ N oligonucleotide (no. 8). In the 20-min sample (Fig. 3C), the intensity of NS4 and several other oligonucleotides is increased, but the overall pattern remains basically the same. There is still no evidence of M5 at 20 min. However, by 30 min (Fig. 3D), this oligonucleotide (no. 14) is clearly visible along with at least one other oligonucleotide derived from the M mRNA (no. 15).

Effect of UV irradiation on synthesis of 5′-proximal mRNA sequences. It was reported that a 98% inhibition of total mRNA synthesis by UV irradiation of virions resulted in only a 20% reduction in synthesis of the major leader RNA as well as the additional leader bands (24). We therefore examined the appearance of the 5′ oligonucleotides N2, NS4, and M5 under conditions of UV irradiation in which full-length mRNA synthesis is strongly inhibited. Under these conditions, the 5′-proximal oligonucleotides should become major products of the in vitro transcription reaction if initiation of all mRNAs occurs simultaneously. However, if initiation of each mRNA is dependent on transcription of the preceding gene, then synthesis of all of the 5′-proximal oligonucleotides except N2 should be strongly inhibited under conditions of

UV irradiation in which full-length N mRNA synthesis is inhibited. VSV virions, irradiated for various lengths of time, were allowed to synthesize RNA in vitro. The products of RNase T_1 digestion of this RNA, separated by two-dimensional gel electrophoresis, are shown in Fig. 4.

Those oligonucleotides present in Fig. 4A (10-min UV irradiation) are derived from either the leader RNA (L3) or the 5' half of the N mRNA (oligonucleotides 2 to 7). No oligonucleotides from the 3' end of the N mRNA are present (no. 8, 9, 11, or 12), demonstrating that under these conditions of UV irradiation, full-length mRNA synthesis is completely inhibited. We could not detect either oligonucleotide NS4 or M5 even after exposing the film with an intensifying screen under conditions which should allow detection of oligonucleotides at least 20-fold less abundant than the leader oligonucleotide L3. With a 1.5-min dose of UV irradiation (Fig. 4B) the 3' N oligonucleotides (8 and 9) and NS4 are made in detectable amounts. With only a 40-UV dosage (Fig. 4C) the 5' M oligonucleotide, M5, is visible. Figure 4D shows the fingerprint of the RNA synthesized using VSV virions that had not been irradiated with UV light, showing the complete set of oligonucleotides as in Fig. 2A.

One-dimensional gel electrophoresis of total RNA synthesized by the VSV transcriptase. Because our analysis of the kinetics of appearance of RNase T_1 oligonucleotides indicated sequential rather than simultaneous transcription of 5' mRNA sequences, we attempted to detect the 5' leader RNAs described by Testa et al. (24) using their conditions of one-dimensional gel electrophoresis. Figure 5 shows the products of in vitro transcription reactions separated on a 20% acrylamide gel. These results initially appeared to be similar to those reported by Testa et al. (24) in that we observed two RNA species (RNAs 1 and 2 in Fig. 5) that migrated slightly faster than the major RNA species which we have identified as the leader RNA. These RNAs are present even when heavily irradiated virus is used in the in vitro transcription reaction and no full-length mRNA is synthesized (lane E, Fig. 5). Because our oligonucleotide analysis suggested that only leader RNA and 5' N mRNA sequences should be synthesized after this amount of irradiation, we carried out sequence analysis of the presumed leader RNA and the bands labeled RNAs 1 and 2.

Sequence analysis was performed only on RNA synthesized by virus that had been irradiated with UV light for 10 or 20 min (lanes D and E) because these reactions had the lowest levels of background radioactivity. We have assumed

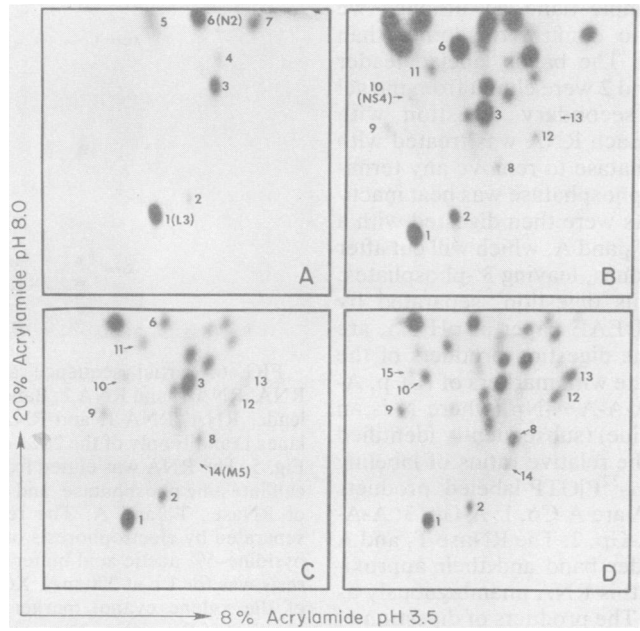

FIG. 4. Two-dimensional gel electrophoresis of RNA synthesized by UV-irradiated VSV virions. UV-irradiated VSV virions were allowed to synthesize RNA in vitro for 2 h in the presence of [α-^{32}P]GTP. This RNA was digested with RNase T_1, and the products were separated by two-dimensional gel electrophoresis as described above. VSV virions were irradiated with UV light as described in the text for (A) 10 min, (B) 1.5 min, (C) 40 s, and (D) 0 s.

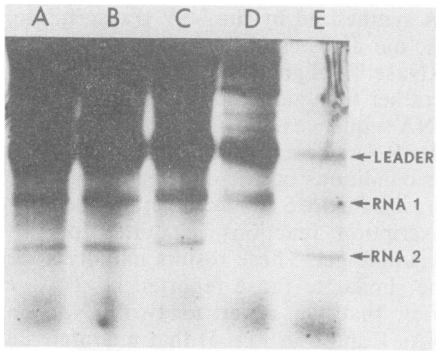

FIG. 5. One-dimensional gel electrophoresis of total RNA synthesized in vitro by UV-irradiated VSV virions. Total RNA synthesized by UV-irradiated virus for 2 h in the presence of [α-^{32}P]GTP was separated by electrophoresis on a 20% acrylamide gel containing 7 M urea. VSV virions were irradiated as described in the text for 0 s (lane A), 40 s (lane B), 1.5 min (lane C), 10 min (lane D), and 20 min (lane E). No attempt was made to separate unincorporated triphosphates (present in the reaction) from the small RNA molecules. Because these unincorporated triphosphates migrate near the bottom of the gel and their presence is independent of UV dosage, a similar amount of radioactivity is present in all five lanes at the bottom of the gel (which is not included in this figure).

that RNAs 1 and 2 are the same RNAs present in the unirradiated sample (lane A) although we have no evidence to confirm this other than position on the gel. The bands labeled leader RNA and RNAs 1 and 2 were eluted from the gel and analyzed by secondary digestion with RNases T_1 and A. Each RNA was treated with calf intestine phosphatase to remove any terminal phosphates, the phosphatase was heat inactivated, and the RNAs were then digested with a mixture of RNases T_1 and A, which will cut after all G, U, and C residues, leaving 3'-phosphates. The products of this digestion, separated by electrophoresis on DEAE paper at pH 3.5, are shown in Fig. 6. The digestion products of the leader RNA comigrate with markers of A-Cp, A-Gp, A-A-Gp, and A-A-A-A-Np (where N is an unidentified nucleoside) (subsequently identified as A-A-A-A-Gp). The relative ratios of labeling expected for the [α-^{32}P]GTP-labeled products from the leader RNA are A-Cp, 1; A-Gp, 3; A-A-Gp, 1; and A-A-A-A-Gp, 2. The RNase T_1 and A products of the leader band and their approximate ratios identify this RNA unambiguously as the leader RNA (6). The products of digestion of [α-^{32}P]GTP-labeled RNA 1 are Up (diffuse but visible on the original autoradiogram), A-Gp, A-A-Gp, and an oligonucleotide that comigrates with A-A-A-A-Np. This latter spot was eluted from the DEAE paper and digested with RNase T_2, and the products were analyzed by electrophoresis at pH 3.5 on Whatman 3MM paper (data not shown). Two products containing nearly equal radioactivity were obtained. These were Up and a spot migrating near Gp (the position of m^{7}GpppAmpAp). Evidence that RNA 1 does in fact contain a cap structure is presented below. The presence of Up indicates that the oligonucleotide A-A-A-A-Np was in fact A-A-A-A-Up (derived from a 3' sequence of A-A-A-A-U-Gp). These products are consistent with RNA 1 being a capped 5' end fragment of the N mRNA about 30 nucleotides long (12, 18) and would not be consistent with RNA 1 being derived from the 5' end of any other VSV mRNA. The major products of digestion of RNA 2 are A-Cp and A-A-

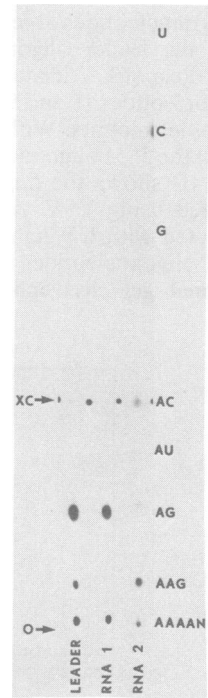

FIG. 6. Partial sequence analysis of the leader RNA, RNA 1, and RNA 2. Bands corresponding to the leader RNA, RNA 1, and RNA 2 were excised from lanes D and E only of the 20% acrylamide gel shown in Fig. 5. The RNA was eluted from the gel, treated with calf intestine phosphatase, and digested with a mixture of RNases T_1 and A. The resulting products were separated by electrophoresis on DEAE paper in 0.5% pyridine–5% acetic acid buffer at pH 3.5. Electrophoresis was for 1 h at 200 mA. XC indicates the position of the xylene cyanol marker, and O indicates the position of the origin. The positions of some markers generated from an RNase A and T_1 digest of RNA are indicated. The oligonucleotides A-A-A-A-Up, A-A-A-A-Cp, and A-A-A-A-Gp are not separable under these conditions of electrophoresis and are therefore indicated as AAAAN.

Gp. Minor spots comigrating with A-Gp and A-A-A-A-Np are also present and are most likely due to background radioactivity in the gel (Fig. 5). These results are consistent with RNA 2 being a 5' fragment of the leader RNA 20 to 25 nucleotides long. These products would not be consistent with RNA 2 being derived from the 5' end of any VSV mRNA.

Because RNA 1 apparently contained a capped 5' end, we carried out a direct analysis for the presence of the cap structure. The leader RNA and RNA 1 ([α-^{32}P]GTP labeled) were both digested with RNases T_1, T_2, and A and treated with bacterial alkaline phosphatase. The products of digestion were separated by electrophoresis on DEAE paper at pH 3.5. This digestion should result in all of the label, except that in a cap structure, migrating as P_i. The results of this analysis are shown in Fig. 7. As expected, the label in the leader RNA is completely converted to P_i. However, RNA 1 is digested to P_i and a product with the same mobility as m^7GpppAmpA, the cap structure found on VSV mRNAs. These results indicate that RNA 1 is a capped RNA containing sequences derived from the 5' end of the N mRNA. RNA 2 is most likely to be a 5' fragment of the leader RNA. These two RNA species could have arisen either by premature transcriptional termination within the leader and N genes or by degradation during the 2-h labeling period. We found no evidence of RNA species derived from the 5'-terminal region of either the NS or M mRNA.

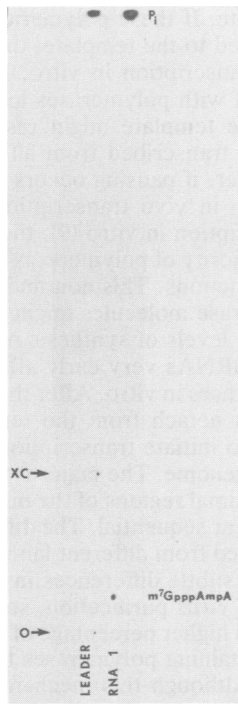

FIG. 7. 5' cap analysis. The leader RNA and RNA 1 (Fig. 5, lanes D and E) were eluted from the gel and treated with bacterial alkaline phosphatase and RNase T_2. The products of digestion were separated by electrophoresis on DEAE paper at pH 3.5 for 3 h at 2,000 V. The position of the origin (O), the xylene cyanol marker (XC), P_i, and the VSV cap structure (m^7GpppAmpA) are indicated.

DISCUSSION

In earlier experiments we examined the kinetics of VSV RNA synthesis by hybridization of mRNA synthesized in vitro to cDNA clones of the VSV mRNAs (9). These experiments confirmed directly that VSV transcription proceeds sequentially and revealed substantial pauses between transcription of adjacent genes. Because the method we used to obtain double-stranded cDNAs results in the obligatory loss of those sequences corresponding to the exact 5' ends of the mRNAs, we could not have detected simultaneous initiation of short 5' mRNA sequences in these experiments. It was therefore not possible, using this hybridization technique, to determine whether or not there was significant synthesis of 5'-proximal VSV mRNA sequences as predicted in the simultaneous initiation model proposed by Testa et al. (24).

To examine the kinetics of synthesis of 5'-proximal VSV mRNA sequences, we analyzed the time of appearance of three RNase T_1 oligonucleotides derived from the 5'-proximal regions of the VSV N, NS, and M mRNAs. These three oligonucleotides were identified as unique spots on two-dimensional gels and should be contained within the respective 5' leader RNAs reported by Testa et al. (24). It should be noted that the NS leader was originally reported to be 28 nucleotides long (based on its electrophoretic mobility) and would therefore not be expected to contain the oligonucleotide NS4 which is derived from nucleotides 15 to 29 of the NS mRNA. However, because the published fingerprint of the NS leader ([α-^{32}P]GTP label) contains a large T_1 oligonucleotide which can only be NS4, we have assumed that this RNA is at least 29 nucleotides long. Furthermore, although RNA 1 was not actually sequenced, it was tentatively identified as being derived from the 5' end of the M mRNA and was approximately 70 nucleotides in length (24). Therefore, oligonucleotide M5 should also be contained within the putative 5' leader RNA identified by Testa et al. (24). Our results show that synthesis of RNA containing these oligonucleotides occurred in a sequential fashion and in the same order as the full-length mRNAs (1, 2, 9). These 5' oligonucle-

otides do not appear until the preceding gene has been transcribed, and their approximate time of appearance is consistent with that predicted from our previous hybridization data (9). N2 is synthesized between 0 and 5 min, NS4 between 5 and 15 min, and M5 between 20 and 30 min after initiation of the in vitro transcription reaction. It should be noted that Testa et al. (24) reported that, using their in vitro transcription system, full-length N, NS, M, and G mRNAs are synthesized within 7 min after initiation of the in vitro reaction. Although the reasons for these major differences in the kinetics of in vitro transcriptions remain unclear, they may be due to differences in the in vitro transcription systems or in the methods of virus purification.

Because our results appeared to rule out significant simultaneous initiation of 5′ leader RNAs for each gene, we reexamined the nature of the several small RNA species synthesized by the VSV transcriptase (24). In addition to the leader RNA, several other small RNAs were detectable by one-dimensional gel electrophoresis of the total RNA synthesized in an in vitro transcription reaction. The synthesis of at least two of these RNAs was also highly resistant to UV irradiation. Partial sequence analysis of these two RNAs indicated that RNA 1 was a 5′ N mRNA fragment containing the 5′ cap structure and that RNA 2 was a 5′ fragment of the leader RNA. It is not surprising that the leader RNA and the 5′ region of the N mRNA should be the major RNA species synthesized by heavily irradiated virus, because these RNAs are transcribed first, and they should therefore be the most resistant to UV inhibition of RNA synthesis. We were unable to detect any short RNAs containing sequences derived from the 5′-proximal regions of any other VSV mRNAs. These results indicate that most VSV transcripts are initiated sequentially, including the synthesis of the 5′-proximal VSV mRNA sequences. We cannot, however, rule out a very low level of independent synthesis of 5′-proximal mRNA regions.

A number of different laboratories have reported synthesis of a small fraction of 5′-triphosphate-terminated RNAs both in vitro and in VSV-infected cells. Lazzarini et al. (11), using γ-thio-ATP to isolate 5′-triphosphate-terminated VSV transcripts, demonstrated that a variable fraction of these transcripts contained the common 5′ mRNA sequence pppAACAG. Because these transcripts could not be chased into full-length mRNAs, it was concluded that they were not precursors of the VSV mRNAs. Naeve and Summers (14) reported the synthesis of 5′-triphosphate-terminated transcripts five to eight nucleotides in length in an incomplete (minus UTP) transcription reaction. These RNAs also appeared to be present in complete (plus UTP) transcription reactions as well and could not be chased into full-length mRNAs. It was suggested that these RNAs might contain sequences derived from the 5′ ends of the mRNAs, but no sequence analysis was reported. Roy and Bishop (23) found 5′-triphosphate-terminated RNAs synthesized in vitro which contained either 5′-ATP or 5′-GTP termini, and Rose (17) reported the presence of a small fraction of polyadenylated RNAs containing either 5′-ATP or 5′-GTP termini in VSV-infected cells. It should be noted, however, that Perrault et al. (15) reported that they were unable to detect any short RNAs other than the leader RNA in an in vitro transcription reaction. From these results it seems clear that the VSV polymerase can initiate RNA chains other than the leader RNA, but it is not clear if such initiation is relevant to the normal transcription process.

If small 5′-triphosphate-terminated RNAs are simply artifacts of the in vitro transcription reaction, the question as to how they arise still remains to be answered. It is possible that during a VSV infection, genomic RNA is packaged into virions with polymerase molecules still on the template. If these polymerase molecules remain attached to the template, they may initiate limited transcription in vitro. Packaging of genomic RNA with polymerases located at random along the template might result in a low level of RNA transcribed from all genomic regions. However, if pausing occurs at intergenic regions during in vivo transcription as it does during transcription in vitro (9), then one might expect the majority of polymerases to be located at the gene junctions. This nonrandom positioning of polymerase molecules might result in low but significant levels of synthesis of the 5′ ends of the VSV mRNAs very early after activation of the polymerases in vitro. After these polymerase molecules detach from the template, they may be able to initiate transcription only at the 3′ end of the genome. The majority of transcription of 5′-proximal regions of the mRNAs would therefore appear sequential. The different observations reported from different laboratories may simply reflect subtle differences in virus strains or methods of virus purification, some of which may result in a higher percentage of packaging of templates containing polymerases located at internal sites. Although this mechanism may explain the simultaneous appearance of transcripts corresponding to the 5′ ends of the mRNAs, it does not explain how RNA molecules of discrete length are generated. The appearance of specific RNA bands would require that there also be preferential sites of transcriptional termination or preferred breakpoints on the RNA transcripts.

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