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5'-Terminal Sequence of Vesicular Stomatitis Virus mRNA's Synthesized In Vitro

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Unmethylated or methylated 12 to 18*S* mRNA's synthesized in vitro by the virion-associated RNA polymerase of vesicular stomatitis virus contain the 5'-terminal hexanucleotide sequence G(5')ppp(5')ApApCpApGp... or m^{7}G(5')-ppp(5')Ap^{m}ApCpApGp..., respectively. The implication of these results in relation to the regulation of transcription in vesicular stomatitis virus is discussed.

Purified vesicular stomatitis virus (VSV) contains a virion-associated RNA-dependent RNA polymerase (3), which synthesizes in vitro multiple mRNA species with sedimentation values of 31*S*, 17*S*, 14.5*S*, and 12*S* (14). The 5'-termini of the latter three species (which when unfractionated sediment heterogeneously between 12 and 18*S*) synthesized in vitro contain the novel structure of G(5')ppp(5')ApAp... (1). When RNA is synthesized in vitro in the presence of the methyl donor *S*-adenosyl-L-methionine (SAM), the 5'-termini of the 12 to 18*S* mRNA's are methylated and consist of m^{7}G(5')ppp(5')-A^{m}pAp... (1, 15). VSV-specific mRNA species of similar sizes and base sequences, which are also methylated and blocked at their 5'-termini (S. A. Moyer, M. J. Grubman, E. Ehrenfeld, and A. K. Banerjee, Virology, in press), are present in VSV-infected cells (13). By in vitro translation, the genetic information of the individual VSV in vitro or in vivo 12 to 18*S* mRNA's have been correlated with the virus structural proteins. The 17*S* and 14.5*S* mRNA's code for the precursor glycoprotein (G) and the nucleocapsid protein (N), respectively, whereas the 12*S* mRNA, which contains two mRNA species of similar molecular weights, codes for the structural proteins NS and M (7, 8). In addition, it was recently demonstrated that only the 17*S* mRNA, which codes for the precursor glycoprotein, is entirely segregated on membrane-bound polysomes in the infected cell (12).

In this communication we have sequenced further from the 5'-terminal ends of both the methylated and unmethylated in vitro mRNA's to answer some of the following questions. (i) Does methylation alter the initiating sequence of the RNA products relative to the 5'-termini of the RNA products synthesized in the absence of methylation, i.e., does it change the initiation site of the polymerase on the genome RNA template? (ii) Are there two 5'-terminal sequences present in the 12*S* RNA corresponding to the two mRNA species detected by in vitro translation (7)? (iii) Does the 17*S* mRNA possess a unique 5'-terminal sequence which may be involved in its selective compartmentalization in infected cells?

The results obtained show, rather interestingly, that the 5'-terminal sequences of the 12 to 18*S* VSV mRNA's are unique in the sense that they probably each contain an identical 5'-terminal hexanucleotide sequence.

MATERIALS AND METHODS

Purification of the virus. The procedures for the growth and purification of VSV (Indiana serotype) in BHK 21/13 spinner cells have been previously described (4).

Synthesis and purification of methylated and unmethylated in vitro mRNA's. RNA was synthesized in vitro using Triton N101-disrupted VSV in standard incubation mixtures as detailed previously (14). [*methyl*-^{3}H]SAM was included in the reaction mixture when methylated mRNA's were synthesized (15). The specific radioactivities of the labeled substrates were as follows: β-γ-[^{32}P]GTP (4.7 Ci/mmol); α-[^{32}P]GTP (7.7 Ci/mmol); α-[^{32}P]CTP (18.2 Ci/mmol); α-[^{32}P]UTP (19.8 Ci/mmol); [^{3}H]SAM (12.6 Ci/mmol). The synthesis was at 30 C for 2 h. The RNA was extracted with phenol and precipitated with ethanol at −20 C. The total 12 to 18*S* VSV mRNA species were isolated and purified by velocity sedimentation as described previously (14, 15).

Purification of the 5'-terminal RNase T_1-fragment. Labeled 12 to 18*S* VSV mRNA's were digested with RNase T_1 (200 units/ml) in a mixture containing 20 mM Tris-hydrochloride (pH 8) and 30 μg of tRNA at 37 C for 2 h. When VSV 12 to 18*S*

mRNA's were labeled with α-[^{32}P]CTP or α-[^{32}P]UTP, the RNA was first selected for poly(A)-containing RNA by oligo(dT)-cellulose chromatography (5) prior to RNase T_1 digestion (see Results). The RNase T_1 digest was extracted with phenol and then adjusted to 0.05 M morpholinium-chloride (pH 8.7), 0.1 M $MgCl_2$, 1.0 M NaCl, and 20% $MeSO_4$ (loading buffer) (16) and loaded on a column (1.1 by 10 cm) of *N*-[*N'*-(*m*-dihydroxyborylphenyl)succinamyl] aminoethylcellulose (DBAE-cellulose). The internal RNase T_1 fragments containing 3'-terminal Gp residues were eluted by loading buffer (6 ml/h flow rate). Portions of the column fractions were dissolved in Aquasol (New England Nuclear) and counted in a liquid scintillation counter. The washing with loading buffer was continued until the radioactivity in the eluted material dropped to background levels. The bound material (3'- and 5'-terminal RNase T_1 fragments) was eluted with loading buffer containing added sorbitol (0.1 M), and portions of the fractions were tested for radioactivity. The fractions containing the labeled material were pooled, diluted fivefold with water, dialyzed exhaustively against distilled water at 4 C, and lyophilized. The 5'-terminal RNase T_1 fragment was then further purified by DEAE-cellulose chromatography in 7 M urea as described previously (2). The fractions containing the radioactive material were pooled, dialyzed against distilled water, and lyophilized.

Enzymatic digestions. The conditions for the enzymatic digestions with RNase A, RNase T_1, nuclease P_1, nucleotide pyrophosphatase, and bacterial alkaline phosphatase have been described in detail elsewhere (1).

Electrophoretic and chromatographic analysis. High-voltage paper electrophoresis was at pH 3.5 in a pyridine:acetic acid:water (1:10:89) system as described previously (1). Descending paper chromatographic analysis was done using isobutyric acid-0.5 M ammonium hydroxide (10:6) as solvent on Whatman 3 MM paper (11). DEAE-cellulose chromatography in 7 M urea and oligo(dT)-cellulose chromatography have been described previously (1, 5, 6).

Chemicals and enzymes. α-[^{32}P]ribonucleoside triphosphates and [*methyl*-^{3}H]SAM were purchased from New England Nuclear, Boston, Mass. β-γ-[^{32}P]GTP was purchased from International Chemical and Nuclear, Irvine, Calif. Bacterial alkaline phosphatase and pancreatic RNase A were purchased from Worthington Biochemical Corp., Freehold, N. J. RNases T_2 and T_1 were from Sankyo Co., Japan, and nucleotide pyrophosphatase was from Sigma Chemical Co., St. Louis, Mo. Nuclease P_1 was from Yamasa Co., Ltd., Japan. DBAE-cellulose and oligo(dT)-cellulose were kind gifts from M. Rosenberg and S. Kerwar, respectively.

RESULTS

Analysis of the 5'-terminal pancreatic RNase A and RNase T_1 fragments of VSV mRNA's. Methylated mRNA's specifically labeled at the 5'-termini were synthesized in vitro in the presence of [^{3}H]SAM (1) and the total 12 to 18*S* RNA (which contains 17*S*, 14.5*S*, and 12*S* mRNA species) was purified and digested either with pancreatic RNase A or RNase T_1. The products of digestion were analyzed by DEAE-cellulose column chromatography. A single ^{3}H-labeled 5'-terminal fragment from RNase A digestion eluted between -5 and -6 charge consistent with the structure as $m^7GpppAp^mApPyp$ (Fig. 1A). Since m^7G carries a single positive charge due to the methylation at the 7 position of the purine ring, the net negative charge of the blocked and methylated RNase A fragment would be expected to be -6 or less, as observed. The 5'-terminal RNase A fragment of the unmethylated 12 to 18*S* mRNA was similarly analyzed using RNA labeled with β-γ-[^{32}P]GTP in vitro (2). A single peak containing ^{32}P eluted between -6 and -7 charge (Fig. 1A, indicated by the arrow), consistent with the oligonucleotide sequence GpppApAp-Pyp. The elution position shifted to one higher negative charge for the unmethylated RNase A fragment compared with the methylated fragment (Fig. 1A). This result is consistent with the neutralization of one negative charge by m^7G only in the methylated RNase A fragment. In similar experiments the RNase T_1 fragment of the methylated ^{3}H-labeled RNA eluted as a single peak between -7 and -8 charge (Fig. 1B) and between -8 and -9 charge for the corresponding fragment of unmethylated mRNA (Fig. 1B, indicated by arrow). These elution positions, together with the data from RNase A digestion, are consistent with the sequences $m^7GpppAp^mApPyp(P^Ay)pGp$ and GpppApAp-Pyp(P^Ay)pGp for the methylated and unmethylated 5'-terminal T_1 fragments, respectively.

Identification of cytosine as the fourth base of 12 to 18S methylated mRNA's. Since RNase T_2 fails to cleave the phosphodiester bond on the 3' side of a nucleoside which contains a 2'-*O*-methylribose substitution, digestion of the methylated VSV mRNA's with RNase T_2 yields a 5'-terminal oligonucleotide with the structure $m^7GpppAp^mAp$ which was previously shown to elute around -5 charge during DEAE-cellulose chromatography (1). The 3'-terminal phosphate of this oligonucleotide is derived from the nearest 3'-neighbor, which must be a pyrimidine as demonstrated above (Fig. 1A). To determine whether the pyrimidine was cytosine or uridine, methylated mRNA's were synthesized in the presence of [*methyl*-^{3}H]SAM and α-[^{32}P]CTP or α-[^{32}P]UTP to find which substrate was the source of this phosphate group. The 12 to 18*S* VSV RNAs were purified and digested with

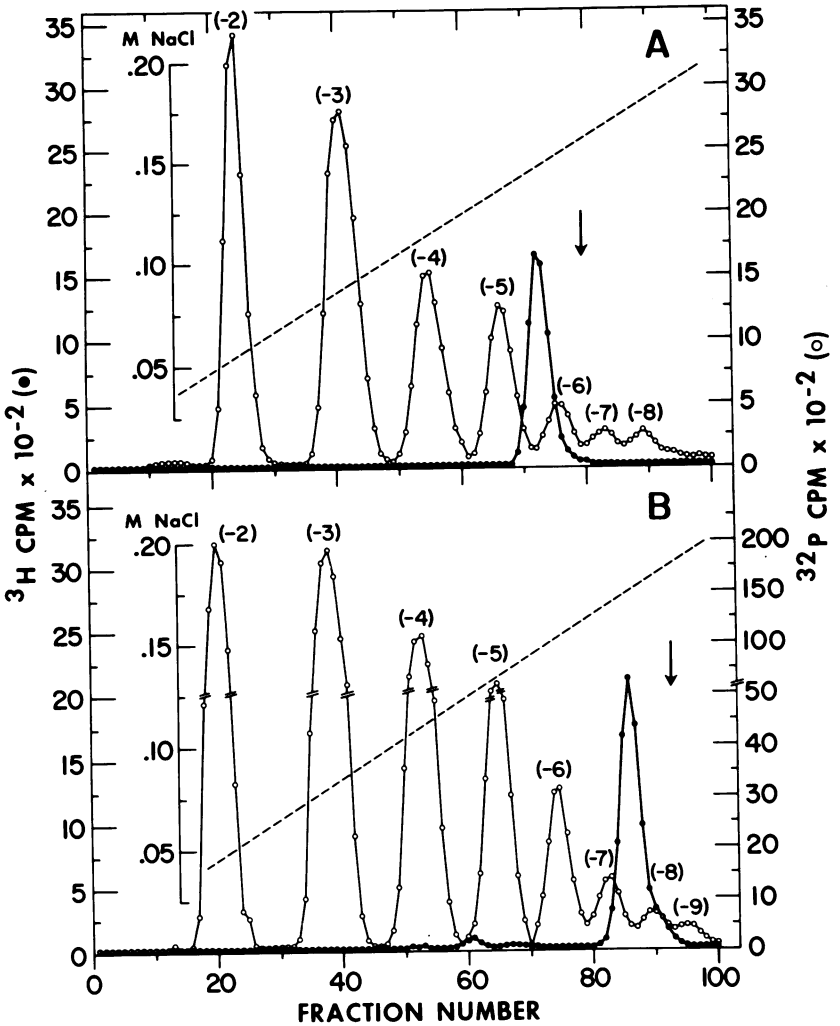

FIG. 1. *DEAE-cellulose column chromatography of the 5'-terminal oligonucleotides of 12 to 18S VSV mRNA species after RNase digestions. Labeled RNA was digested with pancreatic RNase A (100 μg/ml with 1 mg of unlabeled carrier tRNA per ml) for 7 h or with RNase T_1 (200 units/ml with 30 to 100 μg of unlabeled carrier tRNA per ml) for 2 h at 37 C. The digests were extracted with phenol and then with ether, and chromatographed on DEAE-cellulose columns in 7 M urea-50 mM Tris-hydrochloride (pH 8) using an elution gradient of 0.05 to 0.30 N NaCl in the same buffer (6). (A) RNase A digestion of methylated 12 to 18S in vitro RNA labeled with both α-[^{32}P]UTP and [methyl-^{3}H]SAM. The arrow at fraction 80 indicates the elution position of the 5'-terminal fragment from an RNase A digest of unmethylated 12 to 18S RNA labeled in vitro with β,γ-[^{32}P]GTP (determined in a parallel experiment by the salt concentration in the fractions containing the peak of ^{32}P radioactivity). (B) RNase T_1 digest of methylated 12 to 18S RNA labeled in vitro only with [methyl-^{3}H]SAM mixed with a RNase A digest of RNA labeled in vitro only with α-[^{32}P]CTP. The arrow indicates the elution position of the 5'-terminal T_1 fragment of unmethylated 12 to 18S in vitro RNA labeled with β,γ-[^{32}P]GTP determined in a separate experiment as above. Symbols: ●, ^{3}H; ○, ^{32}P; -----, NaCl.*

RNase T_2, and the digests were analyzed by DEAE-cellulose chromatography (Fig. 2). When α-[^{32}P]UTP and [*methyl*-^{3}H]SAM were used as labeled substrates, all the ^{3}H but no ^{32}P eluted at the characteristic position of the 5'-terminal oligonucleotide (Fig. 2A). On the other hand, a peak containing both ^{32}P and ^{3}H radioactivities eluted at −5 charge when the RNAs were labeled with α-[^{32}P]CTP and [*methyl*-^{3}H]SAM (Fig. 2B), suggesting that the fourth base must be cytosine. To show that this [^{32}P]CTP labeled oligonucleotide contained ^{32}P only at the 3'-end and ^{3}H in the blocked 5'-terminal structure, the material eluting at

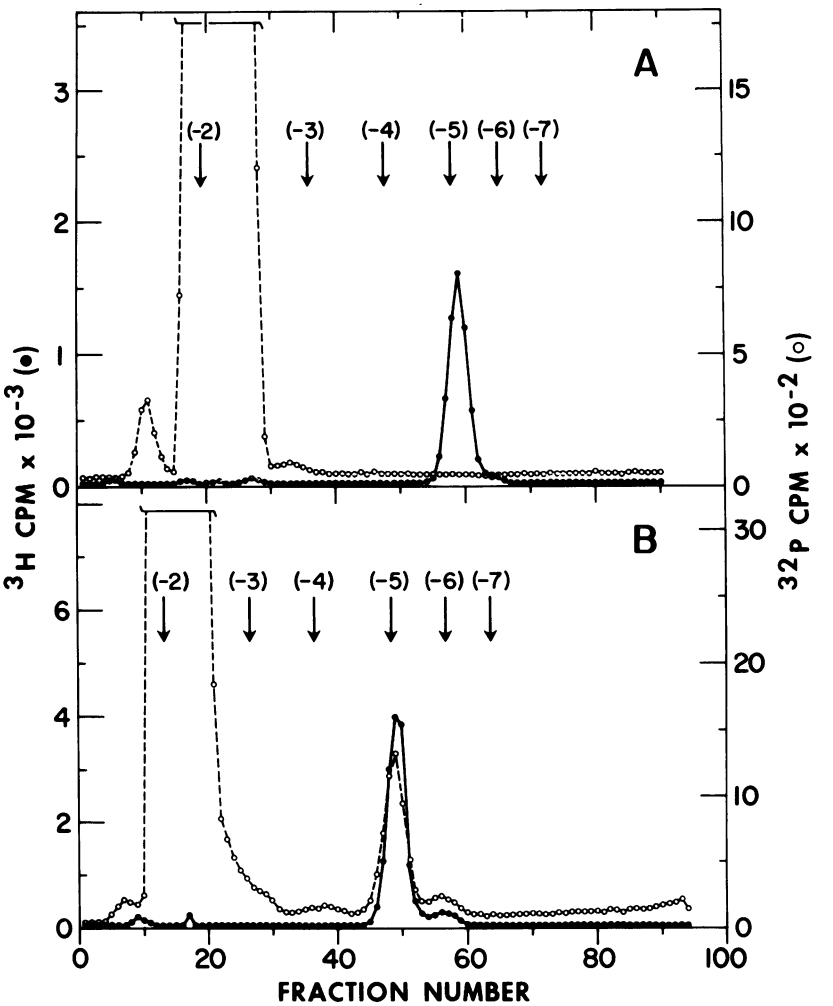

FIG. 2. *DEAE-cellulose column chromatography of methylated VSV RNA species after RNase T_2 digestions. RNA was synthesized in vitro using [methyl-^{3}H]SAM and either (A) α-[^{32}P]UTP or (B) α-[^{32}P]CTP as the labeled precursors. The 12 to 18S RNA was isolated, digested with RNase T_2, and analyzed by chromatography on a DEAE-cellulose column as previously described (1). The arrows and numbers at the top of each graph indicate the elution positions and charges of unlabeled oligonucleotides prepared by digestion of tRNA with pancreatic RNase A. Symbols: ●, ^{3}H; ○, ^{32}P.*

−5 charge (Fig. 2B) was desalted and analyzed by paper electrophoresis. When the material was analyzed without further treatment, both the ^{32}P and ^{3}H comigrated with the pA marker (Fig. 3), the position observed previously for this oligonucleotide (1). When the material was treated with bacterial alkaline phosphatase prior to electrophoresis, however, all of the ^{32}P was released as inorganic phosphate, while the ^{3}H migrated slightly slower (Fig. 3B). These results are consistent with the 5′-terminal sequence of all the 12 to 18S methylated mRNA's being m^{7}GpppAp^{m}ApCp(P^{A}y)pGp....

Isolation of the 5′-terminal fragments of mRNA's by DBAE-cellulose column chromatography. Cellulose derivatives containing covalently bound dihydroxyboryl groups have been successfully used for the isolation and sequence determination of the 3′-terminal regions of isotopically labeled RNA molecules (16, 17). Since VSV mRNA's synthesized in vitro (both methylated and unmethylated) contain free 2′- and 3′-hydroxyl groups at their 5′-ends due to the 5′-5′ linkage of the blocking group (1, 2), DBAE cellulose was utilized to isolate the 5′-terminal fragments of these RNA molecules.

RNA was synthesized in vitro in the presence of [*methyl*-^{3}H]SAM and α-[^{32}P]GTP, and the

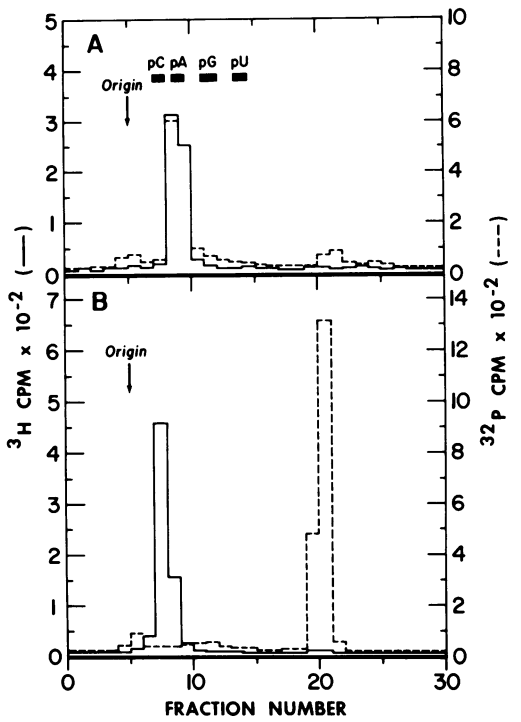

FIG. 3. *Paper electrophoretic analysis of the −5 peak from an RNase T_2 digest of methylated 12 to 18S in vitro RNA. 12 to 18S in vitro RNA was synthesized in the presence of α-[^{32}P]CTP and [methyl-^{3}H]SAM, purified, digested with RNase T_2, and chromatographed on DEAE-cellulose as described in Fig. 2. The −5 peak of ^{32}P radioactivity, corresponding to fractions 46 through 55 in Fig. 2, was located by Cherenkov counting, and these fractions were pooled, dialyzed, and lyophilized. Half of the material was spotted on Whatman 3MM paper without further treatment (A), whereas the other half was digested with alkaline phosphatase before electrophoresis (B). The positions of marker 5'-mononucleotides are indicated by the horizontal bars. Symbols: ——, ^{3}H, - - - -, ^{32}P.*

12 to 18S RNA was purified and digested with RNase T_1. The entire digest was chromatographed on a DBAE-cellulose column. The majority of the ^{32}P was eluted by the loading buffer (Fig. 4). This material presumably contained the oligonucleotides which were terminated with 3'-Gp and will not bind to the column (16), having arisen from the action of RNase T_1 at internal positions. Upon elution with 0.1 M sorbitol, all of the ^{3}H and a portion of the ^{32}P (0.79%) eluted as a single peak (Fig. 4). This material should contain both the 5'- and 3'-terminal T_1 fragments. However, since the 3'-termini of VSV in vitro mRNA's consist of poly(A) (4, 5, 10, 19), the RNase T_1 3'-termini are not labeled by either [*methyl*-^{3}H]SAM or α-[^{32}P]GTP, and only the 5'-termini are detected in the subsequent analyses. The sorbitol-eluted material was dialyzed and further purified by DEAE-cellulose chromatography (Fig. 5). A single peak of radioactivity containing both ^{3}H and ^{32}P eluted between −7 and −8 charge, the position characteristic of the T_1 5'-terminal fragment of the mRNA (see Fig. 1B). Unmethylated mRNA synthesized in vitro in the presence of α-[^{32}P]GTP was processed as above, and the RNase T_1 5'-terminal fragment was similarly purified through the DBAE- and DEAE-cellulose steps (data not shown). The methylated and unmethylated oligonucleotides eluting from the DEAE-cellulose columns at −7 to −8 charge and −8 to −9 charge, respectively, were desalted, lyophilized, and digested with RNase T_2 and analyzed by paper chromatography. In the case of the purified methylated RNase T_1 fragment, almost all of the ^{3}H and 50% of the ^{32}P chromatographed between the Gp (or Up) and Cp markers, the position expected for m^{7}GpppAp^{m}Ap, whereas the remaining 50% of ^{32}P co-chromatographed with the Ap marker (Fig. 6A). These results are

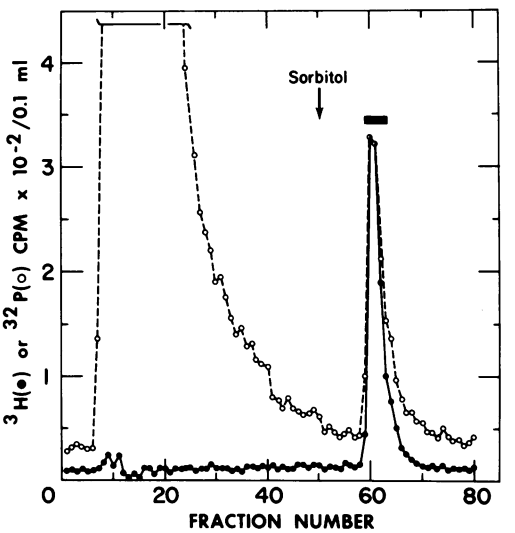

FIG. 4. *DBAE-cellulose column chromatography of RNase T_1 digested methylated 12 to 18S RNA labeled in vitro with [methyl-^{3}H]SAM and α-[^{32}P]GTP. 12 to 18S RNA labeled with α-[^{32}P]GTP and [methyl-^{3}H]SAM was prepared, digested with RNase T_1, and chromatographed on DBAE-cellulose as described in Materials and Methods. Sorbitol (0.1 M) was added to the column buffer at fraction 50 as indicated by the arrow. Portions (0.1 ml) from each 1-ml fraction were counted to locate the peaks of radioactivity. Symbols: ●, ^{3}H; ○, ^{32}P.*

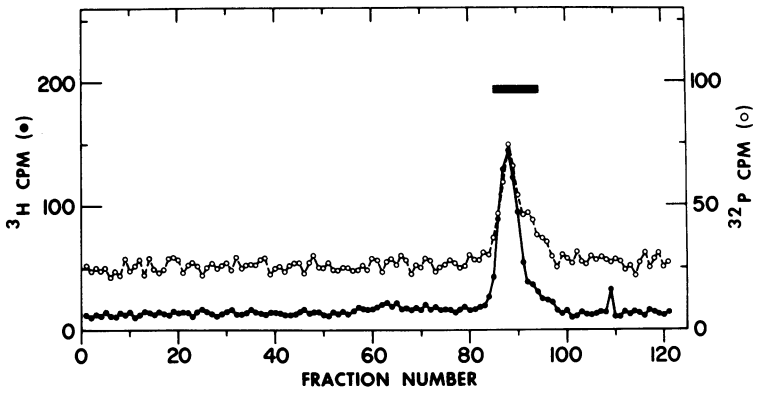

FIG. 5. *DEAE-cellulose column chromatography of 5'-terminal T_1 fragment eluted from DBAE-cellulose. Fractions 59 to 63 of Fig. 4 were pooled (as indicated by the bar) and concentrated as described in Materials and Methods. This material was then chromatographed on DEAE-cellulose as in Fig. 1, and 0.2-ml portions from each 2-ml fraction were counted in Aquasol. Fractions 85 to 94 containing the only significant peak of ^3H or ^{32}P were pooled as indicated by the bar for further analysis. Symbols: ●, ^3H; ○, ^{32}P.*

consistent with the structure of the methylated 5'-T_1 fragment being m^{7}G$\overset{*}{p}$pppAp^{m}ApCpA$\overset{*}{p}$Gp, where the asterisks indicate the ^{32}P derived from -[^{32}P]GTP. RNase T_2 digestion of this oligonucleotide would thus yield two labeled compounds: (i) m^{7}G$\overset{*}{p}$pppAp^{m}Ap containing all of the ^{3}H and half of the ^{32}P, and (ii) Ap containing the remaining half of the ^{32}P by nearest neighbor transfer from pG. By the same argument, the analysis of the unmethylated 5'-T_1 fragment yielded G$\overset{*}{p}$pppAp and A$\overset{*}{p}$ (Fig. 7A), thus having the structure G$\overset{*}{p}$pppAp-ApPypA$\overset{*}{p}$Gp. There is a minor peak of radioactivity (<15%) between fractions 14 to 16 containing both ^{3}H and ^{32}P radioactivity migrating slower than the main peak (fractions 18 to 20) (Fig. 6A). Further analysis of this material revealed that it contained the nuclease P_1 and bacterial alkaline phosphatase-resistant blocked structure, m^{7}GpppA^m. This material may possibly have arisen by opening of the purine ring of the 7-methylguanosine moiety of the blocked 5'-terminal trinucleotide (11).

To demonstrate that the two major peaks in Fig. 6A were indeed m^{7}G$\overset{*}{p}$pppAp^{m}Ap and A$\overset{*}{p}$ generated upon RNase T_2 digestion of the 5-terminal RNase T_1 fragment, these materials were eluted from the paper and analyzed individually by paper electrophoresis. After digestion of the material eluted from fractions 18 to 20 (Fig. 6A) with nuclease P_1, both the ^{3}H and ^{32}P in the presumptive methylated, blocked material migrated in the characteristic position near Ap (Fig. 6B) as shown previously (1). Elution of this material from the paper and treatment with nucleotide pyrophosphatase and bacterial alkaline phosphatase yielded two ^{3}H-labeled compounds which migrated as m^{7}G and A^m upon electrophoresis, whereas all of the ^{32}P was released as P_i (Fig. 6C). Similarly, the ^{32}P-labeled unmethylated, blocked material (fractions 11 to 14) from Fig. 7A migrated between Ap and Gp (Fig. 7B) as shown previously[4] (2). All of the ^{32}P was released as P_i upon treatment with nucleotide pyrophosphatase and bacterial alkaline phosphatase (Fig. 7C). These results indicate that the structures m^{7}G$\overset{*}{p}$pppAp^{m}Ap and G$\overset{*}{p}$pppAp were present in the RNase T_2 digests of the 5'-terminal T_1 fragments of methylated and unmethylated VSV mRNA's, respectively.

The radioactivity migrating with Ap marker (Fig. 6A) was further confirmed to be Ap by electrophoresis. As shown in Fig. 8A, when the material was subjected to electrophoresis it co-migrated with authentic Ap, and it released ^{32}P as P_i when treated with bacterial alkaline phosphatase (Fig. 8B). The same results were obtained when the material with the mobility of Ap in Fig. 7A (released by RNase T_2 from the unmethylated RNase T_1 fragment) was analyzed in this manner (data not shown). These results show that the 5'-terminal T_1 fragment contains two phosphates derived from the α-position of GTP, one recovered in the 5'-terminal pyrophosphate linkage and the other entirely as 3'-AMP. Thus, adenosine is the fifth base in all of the four mRNA species.

Identification of cytosine as the fourth base of the unmethylated 12 to 18S VSV mRNA. VSV mRNA's were synthesized in vitro in the absence of methyl donor but with α-[^{32}P]CTP as the labeled substrate, and the 12 to 18S species were purified. The poly(A) containing

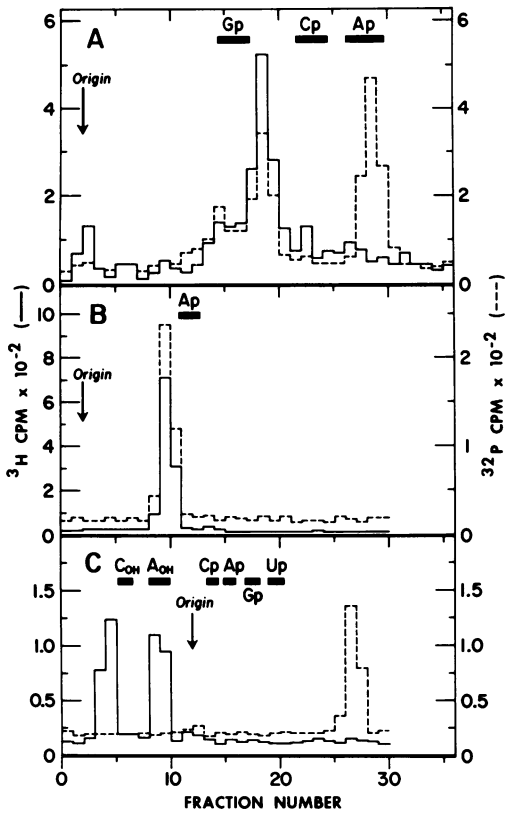

FIG. 6. *Analysis of the methylated 5'-terminal T_1 fragment labeled with α-[^{32}P]GTP and [methyl-^{3}H]SAM. The 5'-terminal T_1 fragment purified sequentially by chromatography on DBAE-cellulose (Fig. 4) and DEAE-cellulose (Fig. 5) columns was digested with RNase T_2, mixed with 3'-nucleotide markers, and analyzed by chromatography (A) on Whatman 3MM paper using isobutyric acid-0.5 M aqueous NH_4OH (10:6) as the solvent. (B) The double-labeled material in strips 18 to 20 of panel A was eluted with water, digested with nuclease P_1, and analyzed by electrophoresis on Whatman 3MM paper. (C) The double-labeled material in strips 9 to 11 of panel B was eluted, digested with nucleotide pyrophosphatase and alkaline phosphatase, and analysed by paper electrophoresis. Conditions for the enzymatic digests have been previously described (1). Chromatography was done for 18 h, and electrophoresis was at 55 V/cm for 60 min (panel B) or 40 min (panel C). Symbols: ——, ^{3}H; -----, ^{32}P.*

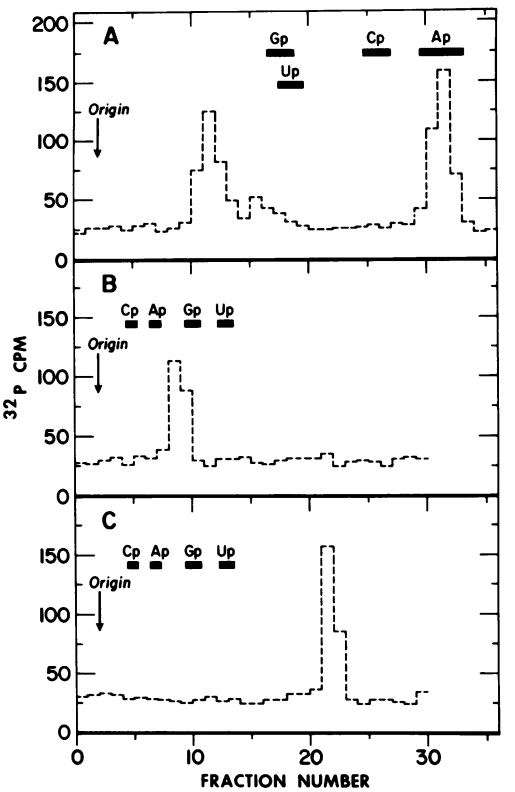

FIG. 7. *Analysis of the unmethylated 5'-terminal T_1 fragment labeled with α-[^{32}P]GTP. Unmethylated 12 to 18S RNA was synthesized with a α-[^{32}P]GTP, and the 5'-terminal T_1 fragment was purified by the same procedures as described in Fig. 4 and 5 for the isolation of the methylated 5'-terminal fragment. (A) The purified 5'-terminal T_1 fragment was then digested with RNase T_2 and analyzed by paper chromatography as described in Fig. 6A. The material in strips 11 to 13 or panel A (from a separate experiment) was eluted with water and (B) one-half was digested with alkaline phosphatase and analyzed by paper electrophoresis, whereas (C) the other half was digested with both nucleotide pyrophosphatase and alkaline phosphatase prior to electrophoresis.*

RNA was purified by oligo(dT)-cellulose chromatography (5). This step was included to avoid any complications arising after RNase T_1 digestion from contaminating oligonucleotides containing CMP residues at the 3'-end of the RNA molecules lacking poly(A) sequences. The α-[^{32}P]CTP-labeled poly(A) containing RNAs were then digested with RNase T_1, and the 5'-terminal T_1 fragment was purified by DBAE- followed by DEAE-cellulose column chromatography as described above. A portion of the RNase T_1 fragment was then digested with RNase T_2 and analyzed by paper chromatography. As shown in Fig. 9A, most of the ^{32}P radioactivity migrated again with Ap. This material was eluted and confirmed to be Ap by paper electrophoresis (data not shown), showing that the ^{32}P from CMP was transferred to AMP. To ascertain that α-[^{32}P]CMP was indeed present in the T_1 fragment, a portion was digested with nuclease P_1 (which yields 5'-monophosphates) and analyzed by paper electrophoresis. Virtually all of the radioactivity mi-

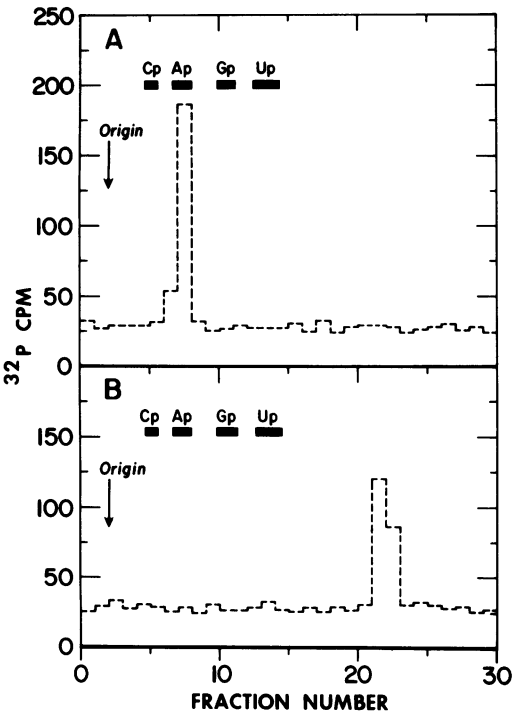

FIG. 8. *Characterization of Ap derived by RNase T_2 digestion of the unmethylated 5'-terminal T_1 fragment labeled with α-[^{32}P]GTP. The material migrating with the Ap marker in strips 31 to 33 of Fig. 7A was eluted with water and analyzed by paper electrophoresis (A). The material in strips 7 and 8 of panel A was eluted, digested with alkaline phosphatase, and again analyzed by electrophoresis (B).*

grated with authentic pC (Fig. 9B). On the other hand, when α-[^{32}P]UTP was used as the labeled substrate and the RNase T_1 fragment was isolated as described above, there was virtually no radioactivity recovered at the characteristic elution position (−8 to −9) in the DEAE-cellulose column (data not shown). There is a minor peak of radioactivity migrating with Gp in both Fig. 9A and 9B. The levels of radioactivity were too low to allow further analysis of these materials. The possible origin of these materials remains unclear at present. However, these results seem to indicate that cytosine is the fourth base in the 5'-terminal RNase T_1 fragment of all of the 12 to 18*S* VSV unmethylated mRNA's as well as in the methylated RNA shown above.

DISCUSSION

The data presented in this paper indicate that the four species present in 12 to 18*S* VSV mRNA synthesized in vitro in the presence or in the absence of the methyl donor SAM contain an identical hexanucleotide sequence at their 5'-termini, GpppApApCpApGp... and m^{7}Gppp-Ap^{m}CpApGp..., for the unmethylated and methylated RNA, respectively. A DBAE-cellulose column, originally used to select the 3'-terminal fragments of RNA chains (17), was used to isolate these 5'-terminal RNase T_1 fragments since they also contain free 2',3'-hydroxyl groups. This procedure, thus, could be effectively used to sequence further from the 5'-termini of both in vitro and in vivo VSV mRNA's, using partial RNase T_1 or RNase A fragments. The 5'-terminal sequence of the 31*S* VSV mRNA species (14) has not been studied as extensively due to lack of sufficient material. Nevertheless, we have recently found that the methylated 31*S* mRNA's made in vitro and in vivo contain the blocked 5'-terminal dinucleotides m^{7}GpppAp^m... and m^{7}GpppAp^{m2}..., respectively (S. A. Moyer and A. K. Banerjee, unpublished observations), similar to the other mRNA species reported previously (1, 13).

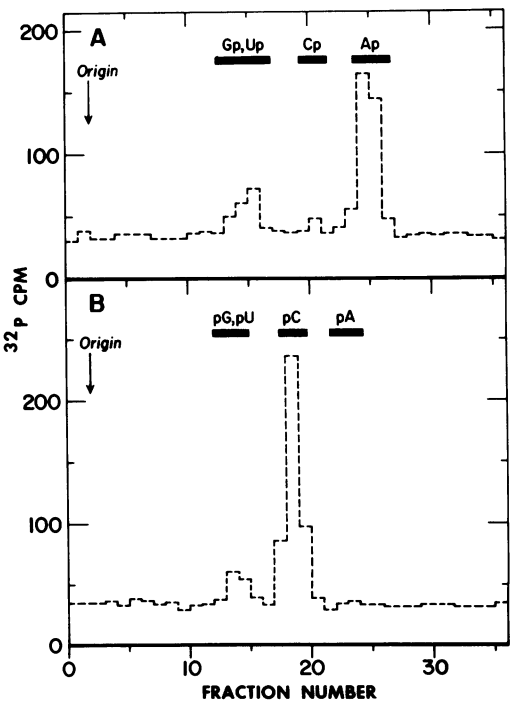

FIG. 9. *Analysis of the unmethylated 5'-terminal T_1 fragment labeled with α-[^{32}P]CTP. Unmethylated 12 to 18S RNA was synthesized with α-[^{32}P]CTP, and the 5'-terminal T_1 fragment was purified as in Fig. 4 and 5. One-half of the purified T_1 fragment was digested with RNase T_2 (A), whereas the remainder was digested with nuclease P_1 (B) and both digests were analyzed by paper chromatography.*

Presence of the same 5'-terminal sequence for both the methylated and unmethylated forms of four of the VSV mRNA species strongly indicates that methylation does not cause any transcriptional alterations in vitro in at least the 5'-terminal portions of the mRNA molecules. Also, it suggests that the mRNA's containing unmethylated and blocked 5'-termini are the intermediates in the eventual methylation of the 5' ends of the mRNA's by the virion-associated methyl transferase (1).

It appears from the results that the 17*S* mRNA species is not different from the other mRNA species with respect to their 5'-terminal sequences, but the following points should be considered in this regard. In our hands, the relative amounts of 17*S*, 14.5*S*, and 12*S* species within the 12 to 18*S* mRNA synthesized in vitro are (in mole percent) 12, 48, and 40, respectively. Thus, if the 17*S* mRNA, which contributes 12% of the 5'-termini, has a 5'-terminal sequence different from the majority of the 12 to 18*S* mRNA molecules, it might not be detected due to the sensitivities of the methods used. However, we feel that it is unlikely, although possible, that a second sequence is present in the in vitro 12 to 18*S* mRNA at this level. Assuming that the 5'-terminal sequences are the same in vivo and in vitro, the compartmentalization of this RNA species in infected cells appears to be a more complicated process than just the recognition by the membrane-bound ribosomes of the 5'-terminal hexanucleotide of the mRNA's. Also, by 5'-terminal sequence determination, we failed to demonstrate the presence of two mRNA species in the 12*S* RNA peak. Evidently other methods have to be employed to achieve this goal.

The 5'-terminal sequences of in vitro synthesized mRNA's usually reflect the sequences of the initiating sites for the polymerase in the template. In the case of VSV, these sequences would be identical, consisting of CpUpGpUpUp... complementary to the sequence GpppApApCpApGp, assuming that the blocking guanyl residue does not base pair and arises by a nontranscriptional mechanism. Since the VSV mRNA's are blocked at their 5'-termini and only the α-phosphate of ATP is incorporated into the pyrophosphate linkage, two modes of synthesis for the mRNA's are possible. (i) The virion-associated RNA polymerase initiates at different sites on the genome RNA to synthesize five different mRNA species in vitro. Therefore, the sequence ...CpUpGpUpUp... should be repeated five times along the genome RNA and should be at least part of the recognition site for the polymerase. Determination of the 3'-terminal sequence of the VSV genome RNA will confirm whether the polymerase initiates any mRNA from the 3'-end of the template or distal to it. (ii) The virion-associated polymerase initiates at a single site at or near the 3'-end, and the individual mRNA's are derived by post-transcriptional cleavage followed by blocking of the new 5'-ends (and adenylation of new 3'-ends). The ...ApApCpApGp... sequence could serve as part of the recognition site for a "restriction" type enzyme for processing and capping. Experiments are in progress to determine which method is used by VSV.

Roy and Bishop (18) have reported that the total product RNA synthesized in vitro by isolated cores of VSV has a variety of 5'-terminal sequences, predominantly initiated with 5'-pppApCpGp... with lesser amounts of 5'-pppGpCp.... In a subsequent report they have shown that 5 to 20% of the transcripts contain unblocked 5'-terminal sequences such as pppApApCpPypGp... in several rhabdoviruses (9). In our in vitro conditions we have failed to detect in 12 to 18*S* mRNA's any 5'-terminal sequences other than the blocked structure GpppApApCpApGp..., or the blocked and methylated structure m^7GpppAp^{m}ApCpApGp..., depending on the conditions of synthesis. The reason for this discrepancy is not clear.

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