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Characterization and Translation of Methylated and Unmethylated Vesicular Stomatitis Virus mRNA Synthesized In Vitro by Ribonucleoprotein Particles from Vesicular Stomatitis Virus-Infected L Cells

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Ribonucleoprotein particles isolated from extracts of vesicular stomatitis virus (VSV)-infected L cells synthesized in vitro four classes of polyadenylated RNA sedimenting at 29*S*, 19*S*, 17*S*, and 13*S*. When synthesized in vitro in the presence of the methyl donor *S*-adenosyl methionine, these RNA species contained the following 5'-terminal structures: (i) $m^7G^{5'}ppp^{5'}AmpAp$ (70%); (ii) $m^7G^{5'}ppp^{5'}AmpAmpNp$ (20%); and (iii) pppAp (10%). In the presence of the methylation inhibitor *S*-adenosylhomocysteine, however, the mRNA contained the 5'-terminal structures $G^{5'}ppp^{5'}Ap$ (80%) and pppAp (20%). The mRNA's synthesized in vitro were translated in the homologous ascites and the heterologous wheat embryo cell-free systems. In both, the products were shown by sodium dodecyl sulfate gel electrophoresis and by immunoprecipitation to contain all five viral proteins, L, G, N, NS, and M. The presumed precursor to the G protein (G*) was also identified by fingerprint analysis. Methylated VSV mRNA was more active in protein synthesis than unmethylated mRNA in both the ascites system and the wheat embryo systems. Addition of *S*-adenosylmethionine stiumulated translation of unmethylated mRNA in the wheat embryo but not in the ascites extract. *S*-adenosylhomocysteine, however, by preventing mRNA methylation inhibited the translation of unmethylated VSV mRNA in both systems. The mRNA methylating activity present in wheat embryo S-30 extracts was recovered in the ribosome-free supernatant fraction (S-150) and was insensitive to the protein synthesis inhibitor pactamycin.

Vesicular stomatitis virus (VSV) is an enveloped virus containing a single-stranded RNA genome and five structural proteins, L, G, N, NS and M (1, 34). Since the virion RNA is of the negative sense, transcription by a virion-associated polymerase must take place before viral protein synthesis can begin (4). With the onset of viral protein synthesis, replication of the 42*S* virion RNA occurs and is accompanied by a parallel increase in mRNA synthesis (18). A ribonucleoprotein (RNP) particle, which synthesizes in vitro polyadenylated RNA species complementary to the VSV genome RNA, has been isolated from the cytoplasm of VSV-infected cells (11, 12, 26). The isolated RNP particles contain the 42*S* virion RNA and at least two VSV-specific proteins, N and L (26). It has been suggested that these RNP particles function in in vivo mRNA synthesis and later form the nucleoprotein cores of progeny virions.

RNA complementary to VSV genome has been synthesized in vitro by virion cores that have been released from whole virions by solubilization with detergents (4) and contain the 42*S* RNA as well as the viral proteins L, NS, and N (9). The product RNA is of the same size classes, 31*S* and 18 to 12*S*, as those of viral mRNA found in VSV-infected cells (4, 24, 25), contains a poly(A) sequence at the 3'-terminus (5, 35), and is "capped" at the 5'-terminus (1). The RNA transcribed by the core can be translated in a cell-free wheat embryo system to give the four VSV-specific polypeptides G, N, NS, and M (7). Both et al. (6) also showed that in the heterologous system, translation of mRNA was dependent on methylation and that the unmethylated mRNA could be methylated in vitro by the wheat embryo extract. They suggested that the methylating activity(s) was associated with ribosomes and was dependent on initiation of protein synthesis (6).

To examine the role of the RNP particles in virus-infected cells, we studied in vitro the synthesis of RNA species by the RNP particles,

as well as the translation of the synthesized mRNA, and compared our results with those obtained for mRNA synthesized in vitro by virion cores. We report here that the RNP particles synthesize in vitro three major species of RNA sedimenting at 19*S*, 17*S*, and 13*S* and a minor species sedimenting at 29*S*. The RNP particles also possess methylating and capping activities and, in the presence of *S*-adenosylmethionine (SAM), the product RNA contains 5′-terminal structures of the type of $m^7G^{5'}ppp^{5'}AmpAmpNp$, $m^7G^{5'}ppp^{5'}AmpApNp$, and pppAp. Analyses of the translation products of the mRNA in the homologous ascites and in the heterologous wheat embryo systems by (i) gel electrophoresis, (ii) immunoprecipitation, and (iii) fingerprint technique show that this RNA directs the synthesis of all five VSV-specific polypeptides. Methylation of the mRNA is necessary for translation in both the plant and the mammalian protein-synthesizing systems, and both systems can methylate in vitro VSV-specific mRNA. We also show that the methylating activity of the protein-synthesizing system is not associated with the ribosomes but is present in the supernatant fraction. Preliminary reports of this work have been presented (14; F. Toneguzzo and H. P. Ghosh, Fed. Proc. **34**:675, 1975).

MATERIALS AND METHODS

Cells and viruses. Plaque-purified VSV (Indiana, HR-LT, obtained from L. Prevec) was grown in L cells in suspension in Joklik modified minimum essential media containing 5% newborn calf serum.

RNP complex. The RNP particle was isolated by a procedure slightly modified from that of Galet and Prevec (11). L cells were infected with VSV as described earlier (32), and at 5 h postinfection were harvested at 4 C, washed with 0.01 M phosphate (pH 7.2) containing 0.15 M NaCl, and then disrupted in 0.01 M Tris (pH 7.8). RNP particles containing polymerase activity and present in the 20,000 × *g* supernatant fraction were pelleted at 150,000 × *g* for 2 h, resuspended in 0.01 M Tris (pH 7.8) containing 10% glycerol and 0.002 M dithiothreitol, and stored at −90 C.

Assay of RNA synthesis. The reaction mixture contained 60 mM Tris (pH 8), 10 mM $MgCl_2$, 50 mM NaCl, 6 mM 2-mercaptoethanol, 2 mM each of ATP, UTP, and CTP, 0.4 mM [^{3}H]GTP (specific activity, 50 μCi/μmol), and the polymerase fraction containing 3 to 3.7 mg of protein in 1 ml of reaction mixture and incubated at 28 C. Aliquots were withdrawn at different intervals and spotted on Whatman 3MM paper disks. The polymerized radioactivity was determined by washing the paper disks with cold 5% Cl_3CCOOH (13).

Assay of methylation. The reaction mixture was identical to that used for RNA synthesis except that [*methyl*-^{3}H]SAM was present. ^{3}H methylated RNA was assayed by the procedure described for RNA synthesis.

Preparation of VSV mRNA. The reaction mixture contained the components as described in the assay procedure. After 90 min at 28 C, the reaction mixture was cooled, sodium dodecyl sulfate (SDS) was added to 0.5%, and the RNA was extracted with a mixture of equal volumes of $CHCl_3$ and phenol saturated with 0.5% SDS-0.05 M Tris (pH 7.5). The extracted RNA was precipitated with 2.5 volumes of ethanol in the presence of 0.4 M LiCl and washed with cold ethanol to remove SDS. The final RNA pellet was dissolved in sterilized water. RNA containing poly(A) was separated by chromatography on an oligo(dT)-cellulose column (3).

Characterization of 5′-terminus. Digestion of the RNA with *Penicillium* nuclease P_1, alkaline phosphatase, and nucleotide pyrophosphatase was carried out as described previously (1). The mixed RNase digestion using RNase T_1 and T_2 and pancreatic RNase was carried out by the procedure of Adams and Cory (2). The β-elimination reaction was carried out as follows (1, 30). RNA synthesized in the presence of [*methyl*-^{3}H]SAM was treated with 0.4 mM KIO_4 in 0.1 M sodium acetate (pH 5.3)-1 mM EDTA and kept at room temperature for 3 h in the dark. Excess KIO_4 was neutralized with 1 μmol of glucose per ml, and the oxidized RNA was recovered by ethanol precipitation. The oxidized RNA was dissolved in 0.33 M freshly distilled aniline (pH 4.5) and kept at 25 C for 4 h and precipitated with ethanol. The β-eliminated RNA was digested with *Penicillium* nuclease P_1 as described earlier, and the radioactive product was identified by electrophoresis.

Paper chromatography and electrophoresis. Radioactive nucleotides and nucleosides were separated by paper electrophoresis on Whatman no. 1 paper by using 0.5% pyridine-5% acetic acid buffer (pH 3.5) at 3,000 V for 2 to 4 h. Separation of the capped oligonucleotides and the 5′-terminal pppNp was obtained by electrophoresis in DEAE-cellulose paper (Whatman DE81) by using pyridine-acetate (pH 3.5) buffer at 3,000 V until the marker blue dye moved to 60 to 70 cm. After electrophoresis the papers were dried and the appropriate nonradioactive marker nucleosides and nucleotides were located under UV light. The papers were cut into strips of 0.5 by 3 cm and counted for radioactivity. Paper chromatography was carried out by using Whatman no. 1 paper and isobutyric acid-0.5 M NH_4OH (5:3, vol/vol) as the solvent.

In vitro translation. The ascites cell-free extract was prepared as described earlier (32). The wheat embryo extract was prepared according to Roberts and Patterson (29). The reaction mixture for the wheat embryo protein-synthesizing system contained 20 mM *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.0), 1 mM dithiothreitol, 90 mM KCl, 3.2 mM magnesium acetate, 1 mM ATP, 20 μM GTP, 10 mM creatine phosphate, 40 μg of creatine phosphokinase per ml, 100 μg of wheat germ tRNA per ml, 20 μM of each unlabeled amino acid, 200 μCi of [^{3}H]leucine or 400 μCi of [^{35}S]methionine per ml, 60 μM spermine, and 400 μl of wheat embryo S-30 per

ml. After incubation at 25 C for 90 min, aliquots were analyzed for hot 5% Cl_3CCOOH-insoluble material (15).

The reaction mixture for the protein-synthesizing system from ascites cells contained 20 mM HEPES (pH 7.6), 100 mM KCl, 3.5 mM magnesium acetate, 10 mM 2-mercaptoethanol, 1 mM ATP, 20 μM GTP, 25 μM CTP, 10 mM creatine phosphate, 40 μg of creatine phosphokinase per ml, 50 μg of rabbit liver tRNA per ml, 20 μM of each unlabeled amino acid, 200 μCi of [^{3}H]leucine or 400 μCi of [^{35}S]methionine per ml, and 350 μl of ascites S-23 per ml. The reaction was incubated at 30 C for 60 to 90 min.

Both SAM and *S*-adenosylhomocysteine (SAH) were neutralized to pH 7.0 for the wheat embryo system and to pH 7.5 for the ascites extract by the addition of appropriate quantities of 1 M HEPES at pH 7.6 and 8.1, respectively. SAM was used at 40 μM and SAH was used at 1 mM in both cases.

Characterization of in vitro translation products: polyacrylamide gel analysis. Slab gel electrophoresis was carried out according to Laemmli (21). Reaction mixtures were diluted with an equal volume of 2× sample buffer containing 0.24 M Tris (pH 6.8), 2% SDS, and 2% 2-mercaptoethanol and were heated to 100 C for 2 min. They were then loaded on a 5% polyacrylamide stacking gel in 0.12 M Tris (pH 6.8)-0.1% SDS. The resolving gel was composed of 10% polyacrylamide in 0.375 M Tris (pH 8.7)-0.1% SDS. The acrylamide-to-bisacrylamide ratio used was 30:0:8. Electrophoresis was carried out at 125 V for approximately 5 h. The gels were then dried without staining and destaining and exposed to X-ray film (Kodak RP/RZ).

Fingerprint analysis. Proteins synthesized in vitro were separated by slab gel electrophoresis and the appropriate bands were sliced, using the autoradiogram as a guide. The protein present in the band was eluted from the crushed polyacrylamide slices by incubation with 0.05 M Tris (pH 8.0)-0.5% SDS at 37 C overnight. The protein was precipitated with 10% Cl_3CCOOH and the pellet was washed with 1 M HCl-acetone (1:40, vol/vol). The isolated protein was oxidized with performic acid and digested with 1 mg of tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin per ml for 16 h at 37 C. An additional 1 mg of trypsin per ml was then added, and the incubation was continued for 4 h. The digest was run on a column (0.9 by 22 cm) containing the cation exchange resin (Aminex A-5), and the tryptic peptides were eluted at 52 C with a gradient of 150 ml of 0.05 M pyridine-acetate (pH 3.1) and 150 ml of 1 M pyridine-acetate (pH 5.0), followed by a gradient of 50 ml of 1 M pyridine-acetate (pH 5.0) and 50 ml of 2 M pyridine-acetate (pH 5.1). Fractions were collected directly into scintillation vials, evaporated to dryness at 90 C, and counted for radioactivity.

Immunoprecipitation. Specific and nonspecific immunoprecipitation was performed by the double-antibody technique. Antiserum against VSV was prepared in rabbits according to Kang and Prevec (19). The cell-free translation mixture (0.02 ml) was precipitated with 0.02 ml of either anti-VSV rabbit serum or normal rabbit serum in the presence of 0.5% Triton X-100 in a final volume of 0.1 ml and incubated at 22 C for 30 min. Two-tenths milliliter of sheep antiserum to rabbit immunoglobulin G was then added, and the incubation was continued at 22 C for 2 h. The reaction was diluted to 3 ml with 0.01 M potassium phosphate (pH 7.4)-0.15 M NaCl-0.001 M EDTA-0.1% Triton X-100, and the protein was recovered by centrifugation at 1,000 × *g* for 15 min. The pellet was washed three times with the dilution buffer, dissolved in 2% SDS-0.2% 2-mercaptoethanol-8 M urea, heated at 100 C for 2 min, and applied to 7.5% polyacrylamide gels (15).

In vitro methylation by wheat embryo extracts. The S-30 preparation was prepared as described previously. The supernatant (S-150) fraction was obtained by centrifuging the S-30 at 150,000 × *g* for 3 h and removing the top two-thirds of the supernatant, being careful to avoid the lipid layer on top. The methylation reaction mixture was the same as that for protein synthesis except that the pH of the HEPES was changed to 7.6 and 200 μCi of [*methyl*-^{3}H]SAM was added per ml. The [*methyl*-^{3}H]SAM was neutralized to pH 7.5 by addition of appropriate amounts of 1 M Tris. Pactamycin was used at a concentration of 5×10^{-6} M. The mixture was incubated at 25 C for 20 min, 0.02-ml aliquots were diluted to 0.5 ml with extraction buffer (10 mM Tris [pH 7.5]-100 mM NaCl-5 mM EDTA-0.5% SDS), 50 μg of wheat germ tRNA was added, and the mixture was extracted with 0.5 ml of phenol saturated with 0.05 M Tris (pH 8.0)-0.5% SDS. The phenol layer was reextracted with 0.5 ml of extraction buffer, and the aqueous layers were combined. The RNA was precipitated with 10% Cl_3CCOOH and filtered through membrane filters (Millipore Corp.), and the radioactivity was counted.

Materials. [8-^{3}H]GTP (9.6 Ci/mmol), [8-^{3}H]ATP (26 Ci/mmol), [*methyl*-^{3}H]SAM (8.5 Ci/mmol), [^{3}H]leucine (55 Ci/mmol), and [^{35}S]methionine (220 to 250 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass.; RNase T_1 and T_2 were from Sankyo Co., Tokyo Japan; *Penicillium* nuclease P_1 was from Yamasa Co. Ltd., Tokyo, Japan. Bacterial alkaline phosphatase and pancreatic RNase were from Worthington Biochemicals Corp., Freehold, N. J.; SAM was from Boehringer Co., New York, N. Y.; SAH and nucleotide pyrophosphatase were from Sigma Chemical Co., St. Louis, Mo. Sheep antiserum to rabbit immunoglobulin G and normal rabbit serum were obtained from J. Gauldie, McMaster University. Trypsin (TPCK treated) was obtained from Worthington Biochemicals Co. Aminex A-5 was from Bio-Rad Laboratories, Richmond. Pactamycin was a gift from the Upjohn Co., Kalamazoo, Mich. Triton X-100 was obtained from Rohm and Hass, Toronto, Canada.

RESULTS

Transcription of VSV RNA and methylation of the mRNA in vitro. The isolated RNP particles could synthesize RNA in the presence of all four ribonucleoside triphosphates. At 28 C the incorporation of [^{3}H]GTP was linear for at least 90 min (Fig. 1a). Velocity sedimentation

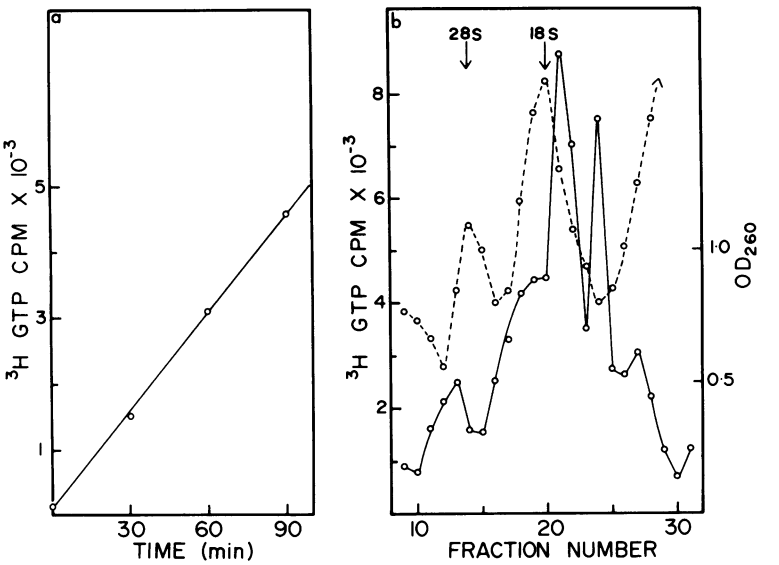

FIG. 1. *Analysis of in vitro-synthesized VSV mRNA by velocity sedimentation on a sucrose gradient. (a) Kinetics of incorporation of [^{3}H]GTP into RNA by the RNP complex at 28 C. (b) The reaction mixture was made to 0.5% SDS and centrifuged at 100,000 × g for 10 h on a 7 to 20% sucrose gradient containing 0.5% SDS-0.1 M LiCl-0.001 M EDTA-0.01 M Tris (pH 7.5). Symbols: (——) 5% Cl$_3$CCOOH-precipitable radioactivity; (- - -) absorbancy at 260 nm profile. Positions of the 18S and 28S rRNA are marked from parallel centrifugation of wheat germ rRNA.*

on a sucrose gradient separated the synthesized RNA into a minor 29*S* species and three major species, 19*S*, 17*S*, and 13*S* (Fig. 1b). The 19*S* RNA species was not clearly separated in this figure but could be resolved into a distinct species by centrifugation for a longer time. The synthesis of RNA at 28 C was quite efficient, and 30 to 35 nmol of [^{3}H]GTP or [^{3}H]UTP could be polymerized in 90 min by RNP complexes containing 1 mg of protein. About 50 to 70% of the RNA synthesized was retained on an oligo(dT)-cellulose column and was eluted with 0.01 M Tris (pH 7.5). The bound RNA fractions on sedimentation analysis showed the presence of all the species present in the unfractionated RNA.

methyl-^{3}H groups donated by *methyl*-^{3}H-labeled SAM were incorporated into acid-insoluble material at a linear rate for up to 90 min by the RNP particle. Sucrose gradient analysis showed that RNA species synthesized in the presence of [*methyl*-^{3}H]SAM and [^{14}C]GTP and sedimenting at 29*S*, 19*S*, 17*S*, and 13*S* all contained both labels. The methylating activity remained associated with the RNP particle containing the transcriptase activity after the RNP particle had been sedimented either through a 20 and 30% glycerol step gradient or partially purified by fractionation on a 15 to 30% glycerol gradient. Gel electrophoresis of the glycerol gradient-purified RNP particles showed the presence of proteins L, N, and NS.

The rate of RNA synthesis was not affected by the presence at concentrations of up to 1 mM of either SAM or SAH, an end product inhibitor of methylation (16). In addition, methylated RNA species synthesized in the presence of 0.1 mM SAM and methyl-deficient RNA synthesized in the presence of 1.0 mM SAH both showed sedimentation patterns identical to that in Fig. 1b. The synthesis of VSV mRNA in vitro, therefore, is not dependent on methylation. In fact, incubation of unmethylated RNA with *methyl*-^{3}H-labeled SAM and the RNP complex showed the incorporation of *methyl*-^{3}H groups into the RNA (Table 1), suggesting that the RNA could also be methylated after it had been completed.

Position of the methyl groups in the RNA molecules. The nature and position of the methylated nucleosides in the RNA synthesized in the presence of [*methyl*-^{3}H]SAM was determined by digesting the RNA with the *Penicillium citrinum* nuclease P_1 (10) followed by alkaline phosphatase. On paper electrophoresis at pH 3.5, the digestion products showed a major radioactive peak moving close to marker pA and a minor peak moving with A (Fig. 2a). The minor peak was identified as Am. The

TABLE 1. *Incorporation of methyl groups into RNA molecules*[a]

RNA added	[*methyl*-^{3}H]SAM incorporated (counts/min)
None	700
rRNA	1,000
tRNA	1,900
VSV mRNA	5,800
VSV mRNA + 1 mM SAH	400

[a] The reaction mixture was the same as described for methylation (see text) except that the ribonucleoside triphosphates were absent. The amounts of rRNA, tRNA, and unmethylated VSV mRNA added were 5 μg, 2 μg, and 1 μg to 0.025 ml, respectively. Aliquots of 0.01 ml were assayed.

absence of any additional ^{3}H products suggests that the methylated RNA did not contain other methylated nucleosides. The major peak of Fig. 2a was identified as the blocked and methylated 5′-terminal sequence containing an internal pyrophosphate linkage by digestion with nucleotide pyrophosphatase followed by alkaline phosphatase. Paper electrophoresis of the digested material showed the presence of two peaks migrating with marker Am and m^{7}G, respectively (Fig. 2b). The peaks were further identified by paper chromatography. These data suggest the presence of a blocked and methylated 5′-terminal nucleotide sequence containing m^{7}G and Am linked by a pyrophosphate linkage.

The 5′-terminal sequence was established by analyzing β-eliminated RNA (30). *methyl*-^{3}H-labeled RNA synthesized in the presence of [*methyl*-^{3}H]SAM was oxidized with HIO_4, and β-elimination was carried out in the presence of aniline. The recovered RNA contained about 50% of the original radioactivity and was digested with *Penicillium* nuclease P_1. Electrophoresis shows a major radioactive peak moving close to marker pppA and a minor peak moving close to pA (Fig. 2c). However, a single radioactive peak corresponding to Am was observed after treatment with alkaline phosphatase. The 5′-terminal sequence is therefore m^{7}G$^{5'}$ppp$^{5'}$Am. The small amounts of Am in Fig. 2a and pAm in Fig. 2c may arise from either internal Am residues or RNA molecules with pAm- in the 5′-termini.

5′-Terminal sequences of RNA synthesized in the presence of SAM or SAH. To find out whether all the RNA chains synthesized contain a capped structure at their 5′ end, we labeled the RNA in vitro, using both [^{3}H]GTP and [^{3}H]ATP in the presence of either SAM or SAH. The methylated or unmethylated RNA was digested with a mixture of pancreatic, T_1 and T_2 RNases, and the digestion products were separated by electrophoresis on DEAE-cellulose paper at pH 3.5. Free 5′-terminal residues will appear as pNp or ppNp or pppNp, whereas the

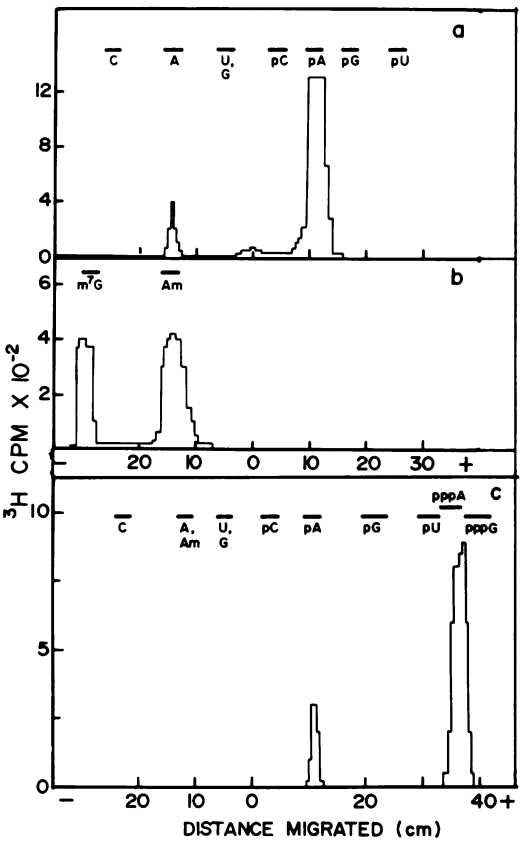

FIG. 2. *Analysis of the blocked and methylated 5′-terminal sequence of methyl-*^{3}*H-labeled RNA.* ^{3}*H-labeled RNA was obtained by using* [methyl-^{3}*H]SAM in the reaction mixture. The RNA was extracted with phenol and the 19 to 12S fraction isolated by sucrose gradient fractionation was used. β-Eliminated RNA was obtained by treating the methyl-*^{3}*H-labeled RNA with* HIO_4 *and aniline. The RNAs were digested with Penicillium nuclease* P_1 *followed by alkaline phosphatase. After analysis by paper electrophoresis on Whatman no. 1 filter paper at pH 3.5 at 3,000 V for 3 h (a), the major peak of radioactive material was eluted from the paper and was digested with nucleotide pyrophosphatase followed by alkaline phosphatase and analyzed by paper electrophoresis (b). Marker nucleosides and 5′-nucleotides were included with each sample, and their positions after electrophoresis are shown. The β-elimination RNA was digested with Penicillium nuclease* P_1 *and analyzed by paper electrophoresis (c).*

capped and methylated 5'-termini will be hydrolyzed to m^{7}G^{5}ppp^5'NmpNp or m^{7}G^5'ppp^5'-NmpNmpNp. Capped but unmethylated 5'-termini will produce G^5'ppp^5'Ap.

The digestion products of the methylated RNA on electrophoresis showed the presence of three peaks (Fig. 3a). Peaks I and II were digested with *Pencillium* nuclease P_1 and alkaline phosphatase and electrophoresed on paper. In both cases, peaks moving close to markers pA and A were obtained (Fig. 3b, c). The peaks co-migrating with marker A in Fig. 3b and 3c were identified as A and Am, respectively, by paper chromatography. The peaks I* and II* were generated from peaks I and II of Fig. 3a by *Penicillium* nuclease P_1 digestion and, therefore, must represent the capped sequence. Hydrolysis of either peak I* and II* with nucleotide pyrophosphatase and alkaline phosphatase, followed by either paper electrophoresis or paper chromatography, showed the presence of only radioactive Am. Since the capped sequence obtained by nuclease P_1 digestion has already been established as m^{7}G^5'ppp^5'Am, both peaks I* and II* may be considered to have the sequence of m^{7}G^5'ppp^5'Am. The absence of any radioactive m^{7}G may be explained by the labile nature of ^{3}H in the C-8 position. The capped sequence of RNA synthesized by virion-associated polymerases from either reovirus or VSV using [8-^{3}H]GTP also did not contain radioactive m^{7}G (A. K. Banerjee and A. J. Shatkin, personal communication).

Since peaks I and II originated by digestion with T_1, T_2, and pancreatic RNases, the oligonucleotides must have the 3'-terminal sequence AmpNp. Also, treatment with nuclease P_1 produced m^{7}G^5'ppp^5'Am and A from peak I and m^{7}G^5'ppp^5'Am and Am from peak II. Therefore, the sequences of peaks I and II may be m^{7}G^5'-ppp^5'AmpAp and m^{7}G^5'ppp^5'AmpAmpNp, respectively.

Peak III of Fig. 3a was identified as pppAp by (i) its electrophoretic mobility relative to pppA (Fig. 3d) and (ii) co-migration with marker A after treatment with alkaline phosphatase.

It appears, therefore, that the 5'-termini of methylated RNA synthesized by the RNP particle contain pppAp-, m^{7}G^5'ppp^5'AmpAmpNp-, and m^{7}G^5'ppp^5'AmpAp in the relative proportions of 10, 20, and 70% (Fig. 3a).

Unmethylated [^{3}H]RNA made in the presence of SAH after hydrolysis and electrophoresis on DEAE-cellulose paper produced two peaks (Fig. 4a). The slower-migrating peak was identified as pppAp. The major peak migrated faster than peak I of Fig. 3a. Paper electrophore-

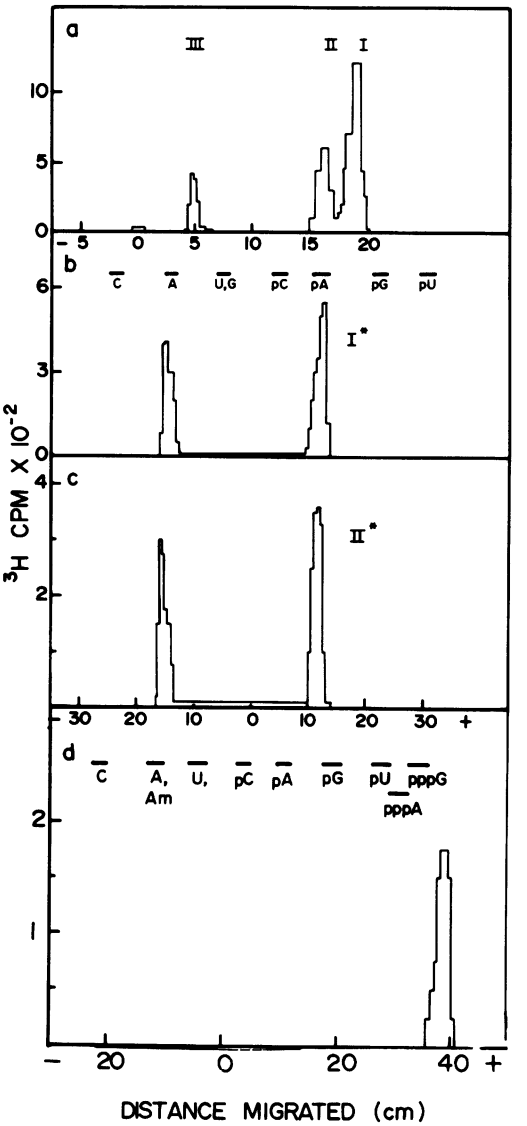

FIG. 3. *Analysis of the 5'-terminal sequences of ^{3}H-labeled RNA synthesized in the presence of SAM. ^{3}H-labeled RNA was obtained by using [^{3}H]GTP and [^{3}H]ATP in the reaction mixture in the presence of 0.1 mM SAM. The product was isolated and digested with a mixture of pancreatic, T_1, and T_2 RNases and electrophoresed on a DEAE-cellulose paper in pyridine-acetic acid (pH 3.5) at 3,000 V for 14 h (a). The blue marker dye moved to about 65 cm from the origin. Radioactivity under the two peaks marked I and II was eluted and separately digested with Penicillium nuclease P_1 followed by alkaline phosphatase and electrophoresed on paper. (b and c) Electropherograms from digested radioactive materials present in peak I and peak II, respectively. (d) Paper electrophoresis of peak III.*

sis of material from this peak after treatment with nuclease P_1 and alkaline phosphatase showed only one peak migrating close to marker pG (Fig. 4b). The products obtained after treatment with nucleotide pyrophosphatase and alkaline phosphatase were identified as G and A, respectively (Fig. 4c). The sequence of the major component obtained from unmethylated RNA could, therefore, be deduced as GpppAp. The relative amounts of GpppAp and pppAp were calculated as 80 and 20%, respectively (Fig. 4a).

Translation in vitro of transcribed mRNA and characterization of the products. Fully methylated RNA synthesized in vitro in the presence of SAM was partially fractionated by sedimentation on a sucrose gradient. The unfractionated RNA and the partially separated 19*S*, 17*S*, and 13*S* fractions were translated in vitro by using protein-synthesizing systems from wheat embryo and ascites cells. The patterns after electrophoresis on 10% polyacrylamide slab gel of the [^{35}S]methionine-labeled products from the wheat embryo system are shown in Fig. 5; the ascites system gave similar results. A protein G*, with a slightly greater electrophoretic mobility than virion G, was detected only in the translation products from the total and 19*S* RNA. Proteins N, NS, and M were synthesized by all the RNA fractions, possibly due to incomplete fractionation and aggregation of the lighter mRNA species. In this slab gel system the proteins N and NS migrated as a single band. After recovery and re-electrophoresis on 7.5% polyacrylamide gels in phosphate buffer (pH 7.2), this band clearly separated into two peaks corresponding to the viral proteins N and NS. G* was detected in only very small amounts, and varying the KCl, spermine, and spermidine concentration did not increase G* synthesis.

G* was identified by fingerprinting as follows. The [^{35}S]methionine-labeled G* band recovered from 10% polyacrylamide slab gel was mixed with [^{3}H]methionine-labeled virion G and digested with trypsin. The pattern of tryptic peptides eluted from a cation exchange column (Fig. 6) shows that the majority of the ^{3}H and ^{35}S peaks overlap. G* therefore appears to be the protein moiety of the virion glycoprotein G, and the difference in electrophoretic mobility may be due to differences in the extent of glycosylation.

The translation products obtained in both the wheat embryo and ascites cell-free systems using unfractionated, methylated RNA were further identified by immunoprecipitation and electrophoresis (Fig. 7). From both systems,

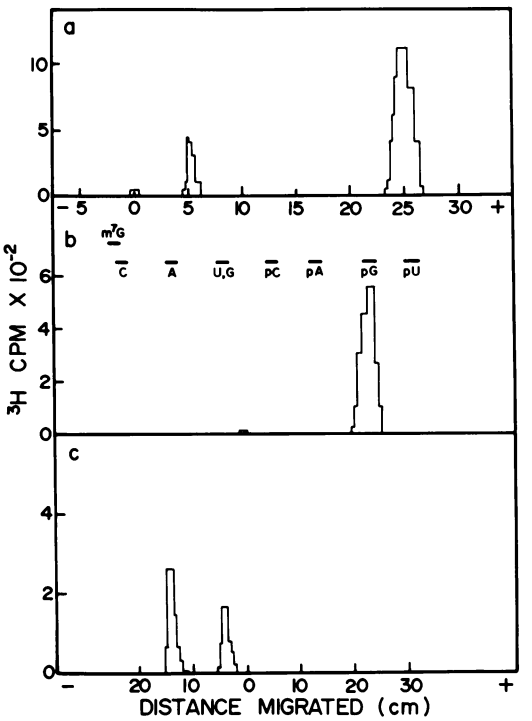

FIG. 4. *Analysis of the 5'-terminal sequences of ^{3}H-labeled RNA synthesized in the presence of SAH. [^{3}H]RNA was obtained by using [^{3}H]GTP and [^{3}H]ATP in the reaction mixture in the presence of 1 mM SAH. The isolated [^{3}H]RNA was digested with a mixture of T_1, T_2, and pancreatic RNases and electrophoresed on DEAE-cellulose paper (a). The radioactivity under the major peak was eluted and digested with Penicillium nuclease and alkaline phosphatase and electrophoresed on paper (b). The radioactive material from the electropherogram (b) was eluted and digested with nucleotide pyrophosphatase followed by phosphatase and electrophoresed on paper (c).*

proteins co-migrating with G*, N, NS, and M were detected with anti-VSV antiserum (Fig. 7a,c) but not with normal serum (Figure 7b,d). An additional small peak was also detected (Fig. 7a,c) which co-migrated with virion L protein. Without immunoprecipitation the background radioactivity observed on slab gels (Fig. 5 and 9) would probably mask the detection of such small amounts of L. Protein bands migrating between NS and M were detected in the gel electropherograms (Fig. 5 and 9) and were presumed to be produced by premature termination. The finding that these bands were specifically precipitated by VSV antibody (Fig. 7) indicates that these polypeptides are also of viral origin.

Translation in vitro of unmethylated VSV

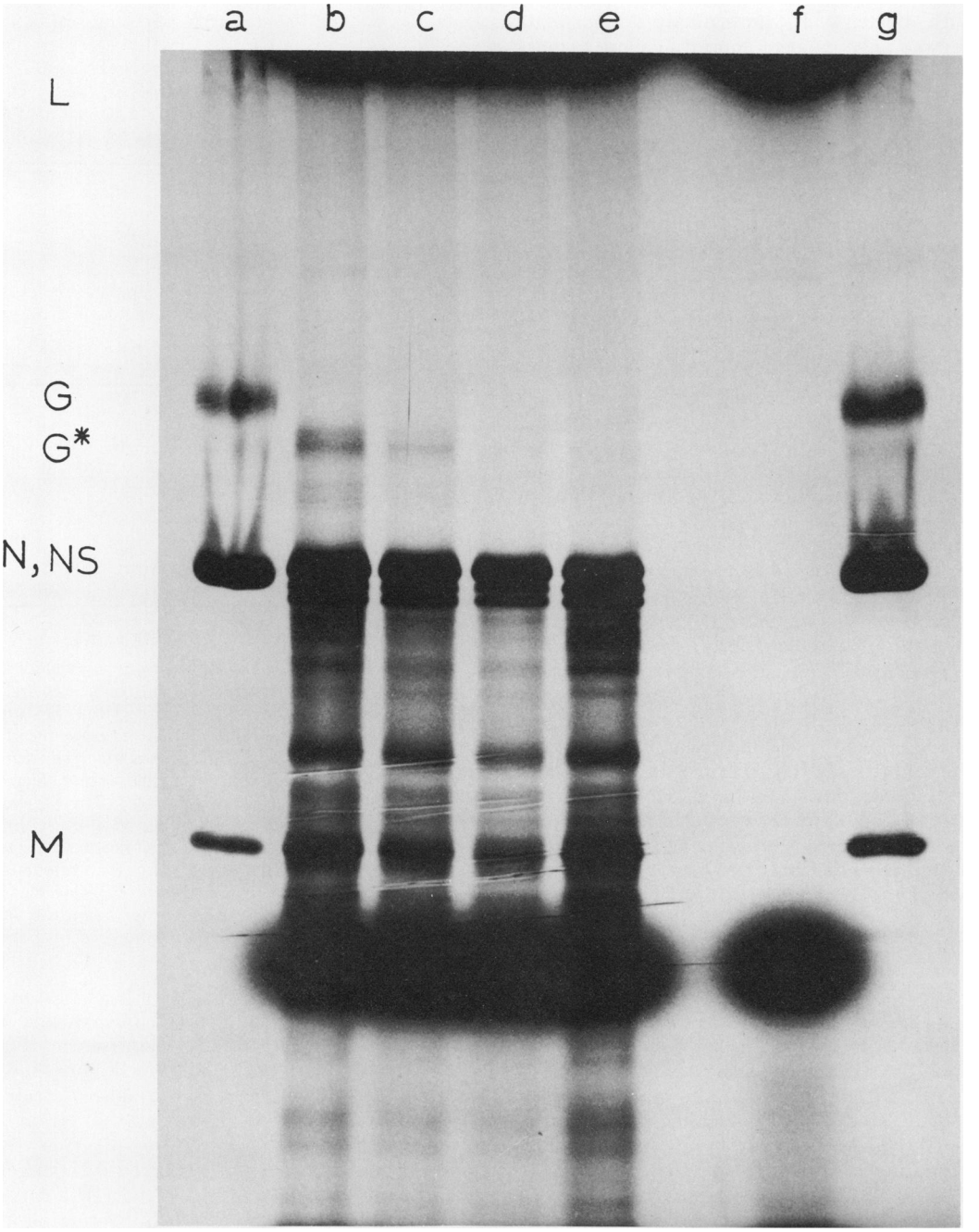

FIG. 5. *Autoradiogram of SDS-polyacrylamide gel of [^{35}S]methionine-labeled products obtained from translation in vitro of VSV mRNA fractions in wheat embryo extracts. Reaction mixtures were diluted with an equal volume of 2× sample buffer and heated to 100 C for 3 min. This mixture was then applied to a 15-cm slab gel containing 10% acrylamide-0.1% SDS in 0.375 M Tris-glycine (pH 8.7) and electrophoresed at 125 V for 6 h. (a and g) VSV virion proteins; (b, c, d, e, and f) proteins synthesized in response to the total RNA, 19S fraction, 17S fraction, 13S fraction, and no mRNA, respectively.*

mRNA. To examine the effect of mRNA methylation on translation, methylated RNA synthesized in the presence of 0.1 mM SAM and methyl-deficient RNA synthesized in the presence of 1.0 mM SAH were prepared. Translation of these two RNAs showed that in both the ascites and wheat embryo systems methylated mRNA was approximately twice as active a messenger as unmethylated RNA (Fig. 8). Since the ribosomal systems used for translation were crude extracts, it seemed likely that they would contain enzymes capable of methylating RNA and so would increase the activity of unmethylated RNA. Translation in the presence of the methylation inhibitor SAH, however, would prevent any mRNA methylation and so should indicate the real activity of unmethylated mRNA. Conversely, addition of SAM to the translation system should allow more complete mRNA methylation and so further increase its activity. These hypotheses were tested in the experiments shown in Fig. 8. In both the wheat embryo and the ascites (Fig. 8) extracts, the translation of fully methylated RNA was unaffected by the addition of either 0.04 mM SAM or 0.04 M SAM plus 1 mM SAH. The addition of 0.04 M SAM plus 1 mM SAH decreased the activity of unmethylated mRNA by 75% in the wheat embryo system (Fig. 8C) and by 50% in the ascites system (Fig. 8B). In the presence of 0.04 mM SAM, however, the activity of unmethylated mRNA in the wheat embryo system was completely restored to that of methylated mRNA (Fig. 8C). Although a slight increase in unmethylated mRNA activity was observed on addition of 0.04 mM SAM to the ascites system (Fig. 8B), the efficiency of translation was still less than that obtained with fully

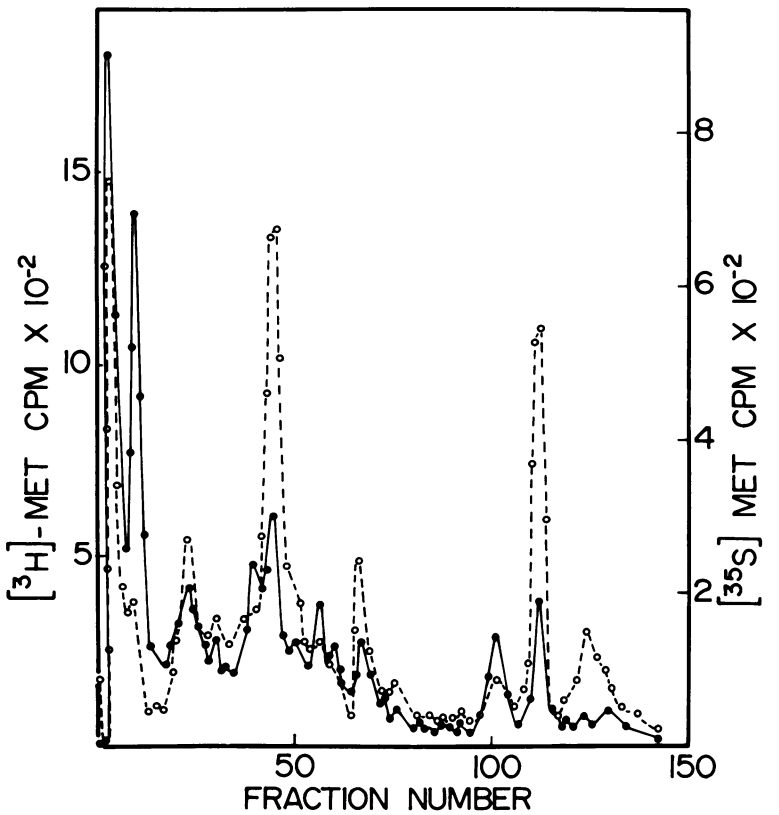

FIG. 6. *Comparison of the tryptic peptides obtained from G* synthesized in vitro with those from virion G. [^{35}S]methionine-labeled G* isolated from 10% preparative slab gels by slicing out the appropriate bands and incubating the slices overnight at 37 C with 0.5% SDS-0.05 M Tris (pH 8.0). 30,000 counts/min of [^{35}S]-methionine-labeled G* was mixed with 120,000 counts/min of [^{3}H]methionine-labeled virion G, isolated from intact virions by treatment with 2.5% Triton X-100 in 0.01 M HEPES (pH 7.6) (9). The mixture was digested with 1 mg of trypsin (TPCK treated) per ml for 16 h at 37 C, and the tryptic peptides were analyzed as described in the text. Symbols: (- - - -) ^{3}H radioactivity; (——) ^{35}S radioactivity.*

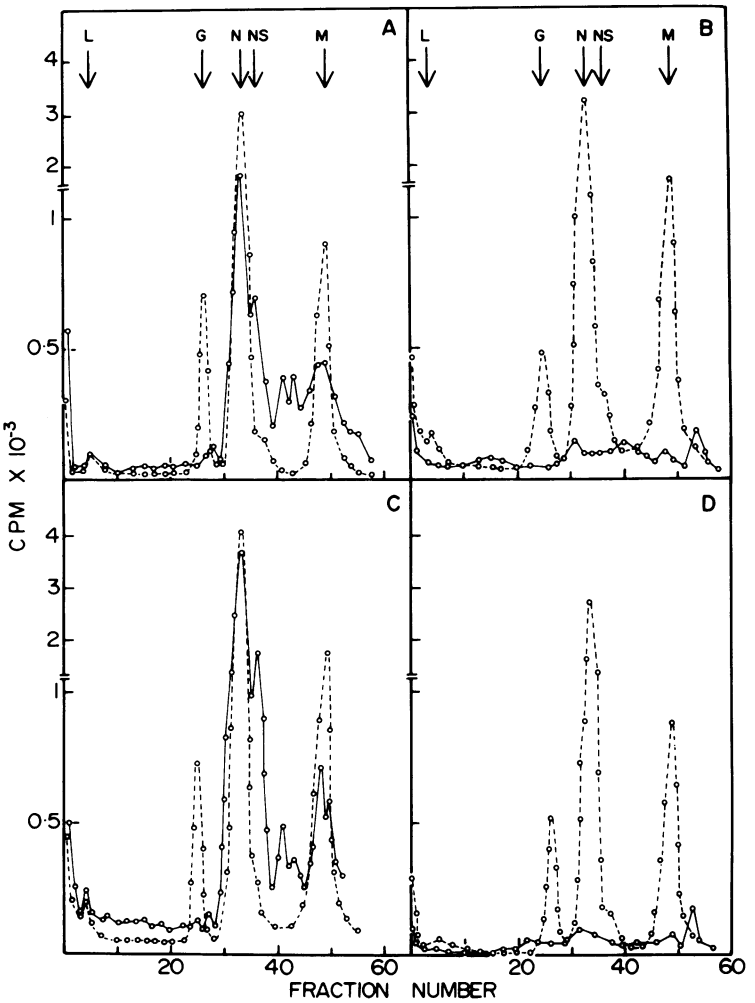

FIG. 7. *Immunoprecipitation of in vitro translation products of VSV mRNA in ascites and wheat embryo protein-synthesizing system. Translation products labeled with [^{35}S]methionine and obtained from the wheat embryo system (0.025 ml; 200,000 counts/min) or the ascites system (0.05 ml; 300,000 counts/min) were mixed with either 0.02 ml of VSV antiserum or 0.02 ml of normal rabbit serum and incubated at 22 C for 30 min. 0.200 ml of sheep anti-rabbit immunoglobulin G was added, and the reaction was incubated at 22 C for another 2 h. The precipitated protein was mixed with ^{3}H-labeled VSV proteins and electrophoresed on cylindrical 7.5% polyacrylamide gels. Symbols: (- - - -), ^{3}H radioactivity; (——) ^{35}S radioactivity. (A and B) Products from wheat embryo translation system precipitated by VSV antiserum and normal rabbit serum, respectively. (C and D) Products synthesized by the ascites extract and immunoprecipitated with VSV antiserum and normal rabbit serum, respectively.*

methylated mRNA. The data thus show that methylation of mRNA is required for its translation in both animal and plant systems.

Analysis of the products synthesized in the presence of SAM and SAH. To determine whether SAM and SAH affect the nature of products translated, the translation products of the methylated and unmethylated mRNA's in the ascites and wheat embryo protein-synthesizing systems were analyzed on 10% polyacrylamide slab gels. The autoradiogram (Fig. 9) shows that there were no differences in the polypeptides synthesized by the methylated or unmethylated mRNA's in the ascites protein-synthesizing system. Presence of SAM or SAM plus SAH also had no effect on the nature of the products synthesized. Similar observations were also made with wheat embryo system.

Methylation in vitro by the protein-synthesizing system. To show directly that the trans-

lation systems are able to methylate RNA, we studied the RNA-dependent incorporation of *methyl*-^{3}H groups donated by [methyl-^{3}H]SAM. Both a wheat embryo extract (S-30) and a supernatant (S-150) fraction were used to methylate VSV mRNA. Addition of unmethylated RNA resulted in a twofold stimulation of *methyl*-^{3}H incorporation into acid-insoluble material by the S-30 fraction and a 10-fold stimulation by the S-150 fraction (Table 2). Addition of 1 mM SAH completely prevented methyl group incorporation. Pactamycin, an inhibitor of initiation of protein synthesis when present at a concentration of 5×10^{-6} M, had no effect on mRNA methylation. The results thus show that methylation of RNA does not require ribosomes and is independent of initiation of protein synthesis.

DISCUSSION

The RNP particle isolated from the cytoplasm of VSV-infected L cells synthesizes in vitro a small but reproducible amount of 29*S* mRNA as well as larger amounts of 19*S*, 17*S*, and 13*S* mRNA species. All four mRNA species are methylated when synthesized in the presence of SAM, although RNA synthesis itself is not dependent on methylation. The presence of 1 mM SAH, which completely inhibits methylation, has no effect on the rate of transcription, and the RNA synthesized contains all the species in amounts similar to those present in fully methylated RNA. The intracellular RNPs, therefore, differ from the virion-associated polymerase in this respect, for Rhodes et al. (28) found that the latter was inhibited by 34% in the presence of 0.68 mM SAH and the mRNA synthesized contained lower amounts of the 12*S* component.

The methylation is specific for the 5′-terminus of the RNA molecules synhesized in vitro by RNP particles. Nucleotide sequence analysis shows that in the presence of SAM, the RNA molecules are synthesized with 5′-terminal structures of the types (i) pppAp, (ii) m^{7}G^5′ppp^5′AmpAp, and (iii) m^{7}G^5′ppp^5′AmpAmpNp present in the respective proportions of 10, 70, and 20%. Analysis of the polysomal RNA from VSV-infected cells by J. Rose (J. Biol. Chem., in press) shows the presence of four populations of mRNA's containing the 5′-terminal sequences pppGp (10%), pppAp (10%), m^{7}G^5′ppp^5′AmpApCp (50%), and m^{7}G^5′ppp^5′AmpAmpCp (20%). On the other hand, Abraham et al. (1) found that the virion-associated polymerase synthesized in vitro RNA species having only the 5′ sequence m^{7}G^5′ppp^5′Amp. In the presence of SAH, the mRNA synthesized in vitro contained only the 5′ sequences G^5′ppp^5′Ap (80%) and pppAp

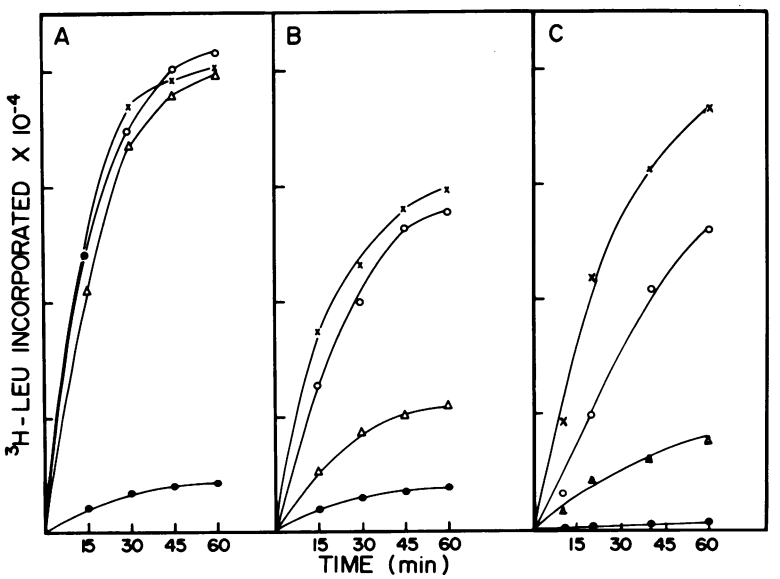

FIG. 8. *Effect of SAM and SAH on translation of methylated and unmethylated VSV mRNA in ascites and wheat embryo. Ascites reaction mixtures (0.06 ml) were incubated with (A) methylated RNA or (B) unmethylated RNA, and wheat embryo extracts were incubated with unmethylated RNA (C). 0.01-ml aliquots were counted for hot Cl_3CCOOH-precipitable radioactivity. Symbols: ●, −mRNA; ○, +mRNA; ×, +mRNA + 0.04 mM SAM; △, + mRNA + 0.04 mM SAM + 1 mM SAH.*

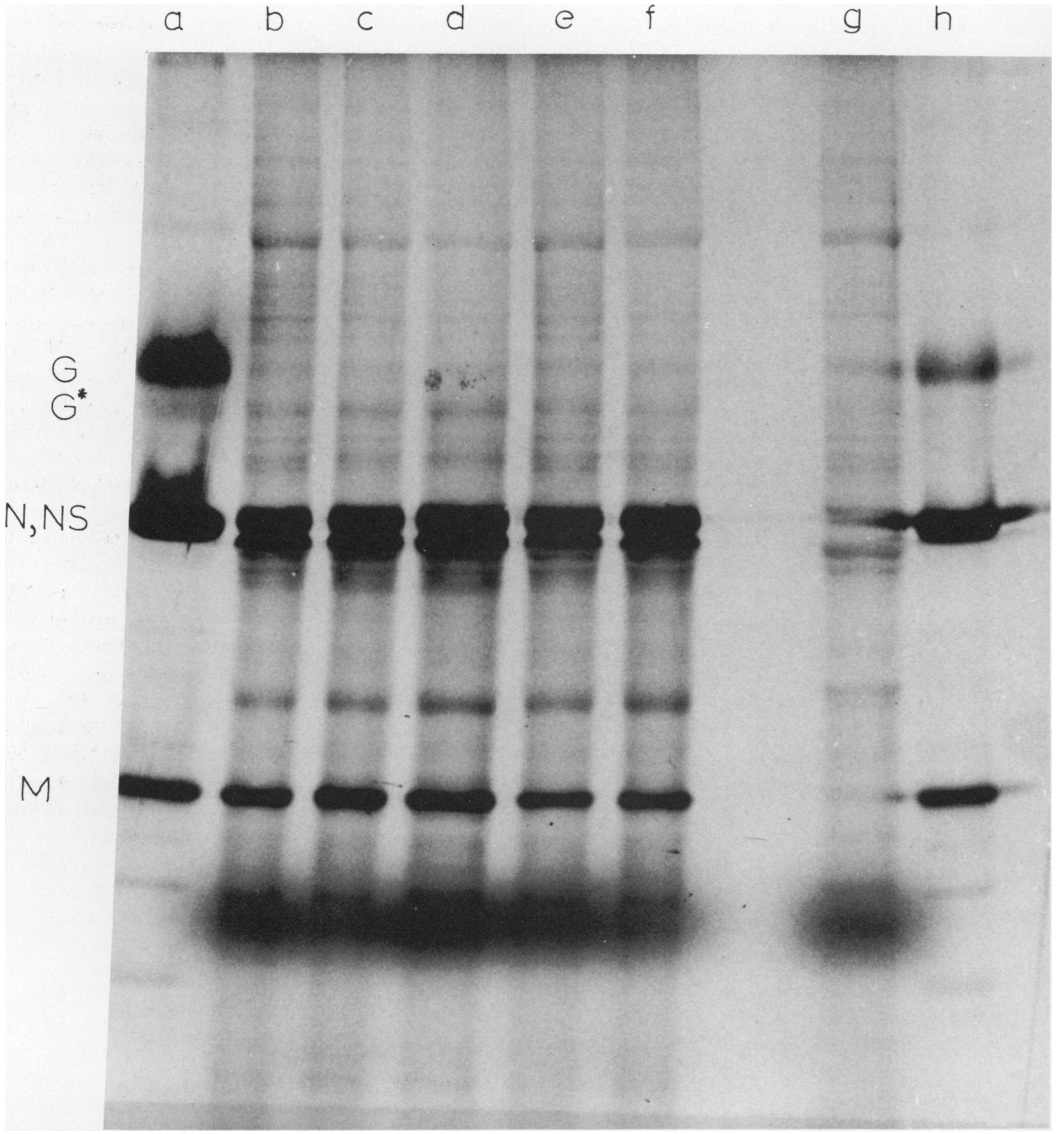

FIG. 9. *Autoradiogram of polyacrylamide gel of [^{35}S]methionine-labeled products directed by methylated and unmethylated VSV mRNA in ascites extracts in the presence of SAM and SAH. Reaction mixtures were applied to 10% polyacrylamide slab gels and electrophoresed. (a and h) VSV virion proteins; (b, c, d, e, f, and g) proteins synthesized in response to methylated mRNA, methylated mRNA + 0.04 mM SAM, methylated mRNA + 0.04 mM SAM + 1 mM SAH, unmethylated mRNA, unmethylated mRNA + 0.04 mM SAM, and no mRNA, respectively.*

(20%). The sequence of reactions involved in mRNA methylation may therefore be (i) initiation of RNA synthesis with pppAp; (ii) blocking of the free 5′-triphosphate termini by formation of an internal pyrophosphate linkage with GTP to form G$^{5'}$ppp$^{5'}$Ap; and (iii) methylation to form m^{7}G$^{5'}$ppp$^{5'}$Am. The RNP particles must therefore possess several enzymatic activities including RNA transcription, polyadenylation, blocking (capping) of the 5′-terminal residue, methylation of the N^{7} of guanosine, and ribosyl methylation of the adenosine residue(s) of the capped sequence. All of these enzymatic functions have also been demonstrated in the "core" polymerase isolated from VSV virions (1).

Translation in vitro of the unfractionated mRNA's synthesized by RNP complexes in

homologous and heterologous protein-synthesizing systems shows that the four VSV-specific proteins G, N, NS, and M are synthesized. Partial fractionation and translation of the mRNA indicates that the 19S species is responsible for the synthesis of G*, the protein moiety of virion G. The faster mobility of in vitro-synthesized G was also observed on translation of both polysomal VSV mRNA's (20, 22, 23, 32) and VSV-specific mRNA's synthesized in vitro by the virion polymerase (7).

Morrison et al. (22) showed that the 28S RNA isolated from the polysomes of VSV-infected cells can direct L protein synthesis in vitro. Gel electrophoresis of immunoprecipitation products allowed the detection of a small amount of L protein synthesis on translation of unfractionated mRNA in the ascites and wheat embryo systems. We also detected the synthesis of a protein co-migrating with virion L protein when unfractionated RNA was translated in reticulocyte lysates (data not presented). The mRNA synthesized in vitro by the RNP particle, therefore, codes for all the five known VSV proteins.

These results, in combination with the observed structural similarities between the mRNA synthesized in vitro by RNP particles and VSV-specific mRNA associated with polysomes in vivo, lead us to conclude that the RNP complex can serve as the major transcription unit in the infected cell. The similarities in protein and nucleic acid composition as well as in enzymatic activities between the virion cores and the intracellular RNP imply that the RNP complexes may eventually become encapsulated to form progeny virions.

Translation of methylated and unmethylated VSV mRNA shows that, in the mammalian ascites system as well as in the wheat embryo system, methylated mRNA is more active in protein synthesis. Addition of SAH to both translational systems inhibits only the translation of unmethylated mRNA. Addition of SAM to the wheat embryo reaction mixture restores the activity of unmethylated mRNA to that observed for fully methylated mRNA. In the ascites system, however, addition of varying amounts of SAM does not result in significant stimulation. These results would be expected if the methylating activities in the ascites extract are present in limiting amounts. Alternatively, it may be that the unmethylated mRNA is unstable in the ascites system and is rapidly degraded.

Our studies thus demonstrate that methylation of the mRNA is essential for translation in vitro of VSV mRNA's in protein-synthesizing systems from both mammalian and plant cells. This is in agreement with the findings of Both et al. (6) and Muthukrishnan et al. (27) showing that methylation of VSV and reoviral mRNA are essential for their translation in vitro in a plant protein-synthesizing system.

TABLE 2. *In vitro methylation of VSV mRNA*[a]

Addition	[*methyl*-^{3}H]SAM incorporated (counts/min)	
	Wheat embryo S-30	Wheat embryo S-150
No RNA	1,600	200
+RNA	3,700	2,500
+RNA + SAH	1,500	300
+RNA		
+Pactamycin (5×10^{-6} M)	3,800	2,400

[a] Reaction mixtures (0.025 ml) containing either the wheat embryo S-30 or the S-150 and [*methyl*-^{3}H]SAM (specific activity, 11 Ci/mmol) were incubated at 25 C for 20 min. Aliquots (0.02 ml) were diluted to 0.5 ml with extraction buffer (see text), and the RNA was extracted by the phenol procedure. The aqueous layer was precipitated with 10% Cl_3CCOOH and filtered on a membrane filter, and the filters were counted for radioactivity.

Both the wheat embryo and the ascites extract can methylate the unmethylated mRNA to the biologically active methylated species. Muthukrishnan et al. (27) have shown that the wheat embryo extract can specifically methylate G^5'ppp^5'Np present in unmethylated reo or VSV mRNA to produce methylated mRNA species containing m^{7}G^5'ppp^5'Np-. Both et al. (6) reported that mRNA methylation by the wheat embryo protein-synthesizing system is inhibited by aurintricarboxylate but not by sparsomycin. They suggest that the methylating activity of the protein-synthesizing system may be ribosome bound and dependent upon initiation of protein synthesis. We, in contrast, observe that most of the methylating activity present in the protein-synthesizing extract (S-30) can be recovered in the ribosome-free supernatant fraction (S-150). Also, pactamycin present in a concentration sufficient for inhibition of the initiation of protein synthesis has no effect on the methylation of mRNA. These results suggest that the methylation of mRNA by the enzyme(s), present in the protein-synthesizing system, does not involve ribosomes and initiation of protein synthesis. The observed inhibition of methylation in the presence

of aurintricarboxylate (6) could be due to the direct inhibition of the methylase(s) by aurintricarboxylate.

Sequence analysis of the fragment of brome mosaic virus RNA species 4, which is specifically recognized by wheat embryo ribosomes, shows that the sequence $m^7G^{5'}ppp^{5'}Gp$ is present at the 5'-terminus (8). The isolated $m^7G^{5'}ppp^{5'}Gp$, however, does not bind significantly to wheat embryo ribosomes. This unique capped and methylated 5'-terminal structure found in mRNA's isolated from both plant and animal systems, therefore, may, in conjunction with other structural features, play a role in ribosome recognition and binding to mRNA's.

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