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Giant Heterogeneous Polyadenylic Acid on Vesicular Stomatitis Virus mRNA Synthesized In Vitro in the Presence of S-Adenosylhomocysteine

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An in vitro transcription system in which vesicular stomatitis virus (VSV) mRNA species have been synthesized is described. In addition to purified VSV virions, which contain an RNA-dependent RNA polymerase, this system contained a cytoplasmic cell extract that enhanced correct transcription. Gel electrophoretic analysis of the methylated polyadenylic acid [poly(A)]-containing VSV mRNA produced in this system in the presence of S-adenosylmethionine showed the discrete VSV mRNA species. However, when unmethylated mRNA was synthesized in the presence of S-adenosylhomocysteine, the poly(A)-containing transcripts were large and heterogeneous in molecular weight and did not contain discrete VSV mRNA species. Two-dimensional fingerprint analysis of the methylated and unmethylated products suggested that identical nucleotide sequences were present in the RNAs. Further analysis showed the presence of very large heterogeneous poly(A), 200 to 2,000 nucleotides in length, in the unmethylated transcript. Proof that this large poly(A) was covalently linked to the correct VSV mRNA transcripts was obtained by removal of the poly(A) by hybridization with oligodeoxythymidylic acid and digestion with RNase H. This digestion produced unmethylated VSV mRNA transcripts with the same discrete sizes as the deadenylated RNAs produced from VSV mRNA initially isolated from VSV-infected cells. The results suggest that there is a relationship between methylation at the 5'-end and polyadenylation at the 3'-end of VSV mRNA's. Furthermore, addition of the very large poly(A) does not affect the normal process of sequential transcription of the VSV genome, suggesting that this poly(A) addition is occurring independently of further transcription.

Infection by vesicular stomatitis virus (VSV) involves intracellular synthesis of five polyadenylic acid [poly(A)]-containing mRNA species that are complementary to the single strand of RNA found within VSV virions, and these RNAs encode the five viral proteins (10, 17, 20, 23, 27). Purified VSV virions contain an RNA polymerase activity (4) that will transcribe the entire VSV genome under appropriate conditions (8). Virions also contain enzymes that will add a sequence of about 200 nucleotides of poly(A) to VSV mRNA's (5, 6, 33) and also generate the methylated 5'-terminal structure $m^7G^{5'}$ $ppp^{5'}$ AmpAp on the mRNA's (1).

Little is known about the mechanism of poly(A) addition to mRNA molecules, although most eukaryotic mRNA's and heterogeneous nuclear RNAs (2, 12, 14, 18, 21, 28) contain poly(A) at their 3'-ends. As in eukaryotic cells, poly(A) is thought to be added post-transcriptionally to VSV mRNA because the genome RNA does not contain polyuridylic acid sequences sufficient to encode the poly(A) (22). Viral transcription may provide a relatively simple model system for studying the mechanism of poly(A) addition.

While we were studying the synthesis and methylation of VSV mRNA in vitro, we found that the presence of S-adenosylhomocysteine (S-Ado-Hcy), which is required to prevent methylation in our in vitro system, resulted in the synthesis of large heterogeneous transcripts that contained normal VSV mRNA sequences. We show here that these transcripts were generated by the addition of very large poly(A) to normally sized, but unmethylated, VSV mRNA's. These results suggest a relationship between methylation of and poly(A) addition to VSV mRNA.

MATERIALS AND METHODS

Virus and cells. BHK-21 cells adapted to Spinner culture were used for virus growth and for preparation of ^{32}P-labeled viral mRNA markers as described

previously (26). Cloned VSV, Indiana serotype, was used for preparation of virus stocks. Two purification procedures were employed for virus that was used in transcription reactions in vitro. One of these procedures has been described previously (6). In addition, we purified virus through two cycles of the procedure described by Banerjee et al. (5), including the bentonite treatment of the virus. Neither procedure yielded virus that in the absence of added cytoplasmic extract would synthesize greater than 10% of the in vitro product RNA as the correct mRNA species. However, these virus preparations and virus purified as described below produced greater than 80% of the product RNA as the correct mRNA species when synthesis was carried out in the presence of cell extract. Unless stated otherwise, all experiments employed virus that had been purified simply by centrifugation of the culture medium at 17,000 rpm for 1.5 h in a Beckman 19 rotor, resuspension of the pellet in TE (10 mM EDTA-10 mM Tris-hydrochloride [pH 8.0]), sonic treatment for 4 s, and sedimentation on a 15 to 40% (wt/vol) sucrose-TE gradient for 1.5 h at 21,500 rpm in a Beckman SW27 rotor. The virus band was removed, diluted twofold with TE, pelleted for 1.5 h at 30,000 $\times g$, and resuspended at a protein concentration of 1 to 5 mg/ml in TE containing 50% glycerol. Virus stored at −20°C in this solution for up to 8 months has shcwn less than 10% loss of either titer or transcriptase activity.

Synthesis of VSV mRNA in vitro. Reactions for synthesis of VSV mRNA in vitro contained purified VSV virions at a concentration of 100 μg of protein per ml, 50 mM Tris-hydrochloride (pH 8.0), 100 mM NaCl, 4 mM dithiothreitol, 0.025% Triton N-101, 1 mM ATP, 1 mM CTP, 40 μM UTP, and 80 μM GTP ([α-^{32}P]GTP specific radioactivity of 3.12 Ci/mmol). For [α-^{32}P]ATP labeling, the GTP concentration was increased to 1 mM and [α-^{32}P]ATP was added to a specific radioactivity of 0.18 Ci/mmol and a concentration of 1 mM. All reactions also contained either 1 mM *S*-Ado-Hcy or 1 mM *S*-adenosylmethionine (*S*-Ado-Met) as indicated, as well as cytoplasmic cell extract. Reactions (usually 0.09 ml) were started after 5 min of preincubation at 30°C by the addition of 0.01 ml of cytoplasmic cell extract (20 mg of protein per ml) containing 50 mM $MgCl_2$. Reactions were incubated for 2 h at 30°C unless otherwise indicated and stopped by the addition of sodium dodecyl sulfate and sodium acetate (pH 5.2) to final concentrations of 1% and 0.2 M, respectively, followed by the addition of 20 μg of carrier tRNA. The sample was extracted with an equal volume of phenol-chloroform (1:1) and centrifuged at 10,000 $\times$ g for 5 min, and the aqueous layer was removed. The phenol-chloroform layer was reextracted, and the aqueous layers were pooled and precipitated with 2 volumes of ethanol. The RNA pellet was resuspended in TE, dimethyl sulfoxide was added to a final concentration of 90%, and the RNA was denatured by incubation for 15 min at 55°C. The sample was again precipitated by the addition of ethanol and 0.1 M sodium acetate, resuspended in 0.5 ml of a solution containing 10 mM Tris-hycrochloride (pH 7.4), 0.4 M NaCl, and 0.02% sodium dodecyl sulfate, and bound to oligodeoxythymidylic acid [oligo(dT)]-cellulose (0.05 g in a Pasteur pipette column). The column was washed with 5 ml of the binding buffer, followed by elution of the poly(A)-containing RNA with 0.1 mM EDTA (pH 7.5). A total of 80% of the product RNA synthesized in the presence of cell extract was retained by the oligo(dT)-cellulose column. All in vitro reactions showed an initial 5-min lag period during which the rate of synthesis increased and then was linear for at least 2 h. Approximately 5 to 10% of the [α-^{32}P]GTP or 0.4% of the α-ATP was incorporated into RNA in this time.

Preparation of cell extracts. Cytoplasmic cell extract was prepared basically as described by Kerr et al. (19). Ascites cells were centrifuged at 1,000 $\times$ g for 5 min and then washed twice with 0.8% NaCl-0.02% KCl-10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.2. Packed cells were then resuspended in 1.5 times their volume of 10 mM HEPES (pH 7.5)-10 mM KCl-1.5 mM magnesium acetate, and 7 mM β-mercaptoethanol was added. This mixture was then centrifuged at 10,000 $\times$ g for 10 min, and the supernatant was recovered. ATP, GTP, CTP, and creatine phosphate were added to final concentrations of 1, 0.1, 0.6, and 10 mM, respectively, and creatine kinase was added to a final concentration of 160 μg/ml. This mixture was incubated for 45 min at 37°C, filtered through plastic mesh, and dialyzed for a total of 6 h at 4°C against three changes of 10 mM HEPES (pH 7.5), 90 mM KCl, 1.5 mM magnesium acetate, and 7 mM β-mercaptoethanol. The dialyzed material was centrifuged to remove debris and stored in small samples at −70°C. The procedure used for a preparation of BHK cell extract was much simpler and appeared to give an equivalent increase in the size of the in vitro product, although we have not tested it extensively. This extract was prepared from 1 liter of BHK-21 Spinner cells (approximately 2 $\times$ 10^8 cells) that were pelleted, washed as above, and resuspended in 10 mM HEPES (pH 7.5)-10 mM KCl-1.5 mM magnesium acetate-7 mM β-mercaptoethanol-0.1% Nonidet P-40. The cells were broken in a Dounce homogenizer and centrifuged as above, and the supernatant was dialyzed immediately without any additions. All operations were performed at 4°C.

Polyacrylamide gel electrophoresis. Product mRNA's and poly(A) derived from these mRNA's were analyzed on either 4% (wt/vol) polyacrylamide slab gels containing 98% formamide, or on 12% (wt/vol) polyacrylamide slab gels containing 7 M urea, 90 mM Tris-boric acid (pH 8.3), 4 mM EDTA, and 0.1% sodium dodecyl sulfate. Samples of the 12% gels were loaded in this buffer containing 10% glycerol and heated at 65°C for 5 min prior to loading. Tracking dyes were bromophenol blue and xylene cyanol FF (Eastman). Polyacrylamide gels containing 98% formamide were described previously (13, 27). Markers of *Escherichia coli* tRNA and 6*S* RNA were obtained from a commercial preparation of *E. coli* tRNA and included on each gel. After electrophoresis, gels were stained in a solution containing 0.2 μg of ethidium bromide per ml, and the positions of the marker RNAs were located by illumination with a short-wave UV Mi-

neralight. Wet gels were covered with Saran Wrap, the positions of markers were indicated by labeling on tape with ink containing ^{35}S, and the gels were autoradiographed for 16 h on Kodak RPR film. Autoradiographs were scanned with a Joyce-Loebl microdensitometer.

Enzymatic digestions. Limit digests with RNases A and T1 to leave poly(A) tracts intact were in 10 μl of a solution containing 20 U of RNase A per ml, 200 U of RNase T1 per ml, 0.1 M NaCl, 0.01 M Tris-hydrochloride (pH 7.5), and a total of 20 μg of RNA (VSV mRNA and carrier tRNA).

Digestions with calf thymus RNase H to remove poly(A) from VSV mRNA were done by the following procedure, a modification of published procedures (30, 34). One microgram (approximately 10^6 cpm) of ^{32}P-labeled VSV mRNA synthesized in VSV-infected cells or 0.1 μg of [α-^{32}P]GTP-labeled VSV mRNA synthesized in vitro (approximately 10^6 cpm) was combined with 10 μg of unlabeled f2 phage RNA carrier and dissolved in a total volume of 10 μl of water. Two microliters of a solution containing 1 mg of oligo(dT)$_{12-18}$ per ml was added, and the mixture was incubated for 10 min at 23°C. One microliter of 0.33 M KCl was then added, and the sample was incubated for 15 min at 23°C. After this incubation, 1 μl of a solution containing 1.9 U of RNase H in 0.6 M Tris (pH 8.0), 0.25 M $MgCl_2$, and 1 mM dithiothreitol was added, and the sample was incubated for 30 min at 37°C. Digested samples were lyophilized and analyzed directly by electrophoresis on formamide-polyacrylamide gels. The presence of the large excess of f2 phage RNA was essential to prevent extensive degradation of the VSV mRNA during digestion of the poly(A). Also, we found it was essential to omit the bovine serum albumin (Pentex) from the enzyme buffer because it contained RNase.

Two-dimensional fingerprint analysis. The homochromatography fingerprinting technique has been described previously (7). Electrophoresis in the first dimension was on cellulose-acetate at pH 3.5 in buffer containing 7 M urea, 10% acetic acid, and 0.5% pyridine. Transfer to the second-dimension polyethyleneimine thin-layer plates employed a concentration technique (32). The second dimension was chromatography in homomixture C (digested in 1 N KOH for 10 min) described by Barrell (7). RNase T1 digestions were for 30 min at 37°C in 3 μl of a solution containing 200 U of RNase T1 per ml, 0.01 M Tris-hydrochloride, 0.001 M EDTA, and 20 μg of RNA (VSV mRNA and carrier tRNA). Approximately 10^6 cpm of ^{32}P-labeled RNA were used in each fingerprint, and the thin-layer plates were autoradiographed on Kodak NS film for 16 h. Samples to be fingerprinted were prepared by phenol extraction and ethanol precipitation as described above and purified free from unincorporated triphosphates by chromatography on Sephadex G-50.

Materials. Radioisotopes were purchased from New England Nuclear Corp., Boston, Mass. RNases T1 and A were from Calbiochem, and calf thymus RNase H was a gift from A. Efstratiadis and L. Villa-Komaroff. Oligo(dT), cellulose (T-3), and oligo(dT)$_{12-18}$ were from Collaborative Research, Waltham, Mass. Carrier tRNA from *E. coli* was purchased from Boehringer Mannheim Corp., New York, and polyethyleneimine (20 by 20 cm) plastic thin-layer plates were purchased from Brinkmann Instruments Inc., Westbury, N.Y.

RESULTS

In our initial studies of VSV transcription directed by the virion-bound polymerase in vitro, we found that virions which had been purified extensively and treated with bentonite to inactivate contaminating nucleases (5) directed the synthesis of an RNA product that was predominantly smaller than VSV mRNA's. Because we were interested in obtaining the majority of the in vitro product as the correct mRNA species, we adopted a procedure of adding a cytoplasmic cell extract to the in vitro reaction to enhance proper transcription. Ball and White (3) have reported the synthesis of a similar small RNA product, using purified virions, and found that addition of cell extract resulted in the synthesis of normal product mRNA (3; Andrew Ball, personal communication).

Effect of cell extract on VSV transcription. Figure 1 shows an autoradiogram of a 12% polyacrylamide gel electrophoretic analysis of [α-^{32}P]GTP-labeled RNA products synthesized in vitro by the virion-bound VSV polymerase. Figure 1A shows the product produced when highly purified virions (5) alone were used in the reaction mixture, and Fig. 1B shows the product produced when virions and a cytoplasmic cell extract were present in the reaction mixture. Without cell extract the product is predominantly small, with an average size close to that of tRNA marker; however, a larger RNA transcript (8% of the total) which did not enter the gel was present. The effect of cell extract on the virion-directed transcription was dramatic; greater than 95% of the product was then too large to enter the gel, and we consistently observed two- to threefold stimulation of GTP incorporation into RNA in the presence of cell extract, although the cell extract alone did not incorporate GTP. As we discuss below, the product RNA synthesized in the presence of cell extract contained the same VSV mRNA species that are found in VSV-infected cells. In contrast, less than 10% of the product made without cell extract comigrates with the VSV mRNA species.

A possible explanation for the small size of the product produced by purified virions is the presence of nucleases that are degrading the product RNA. However, we have not been able to increase the product size by using more extensive purification procedures for the virions, including two cycles of velocity and equilibrium

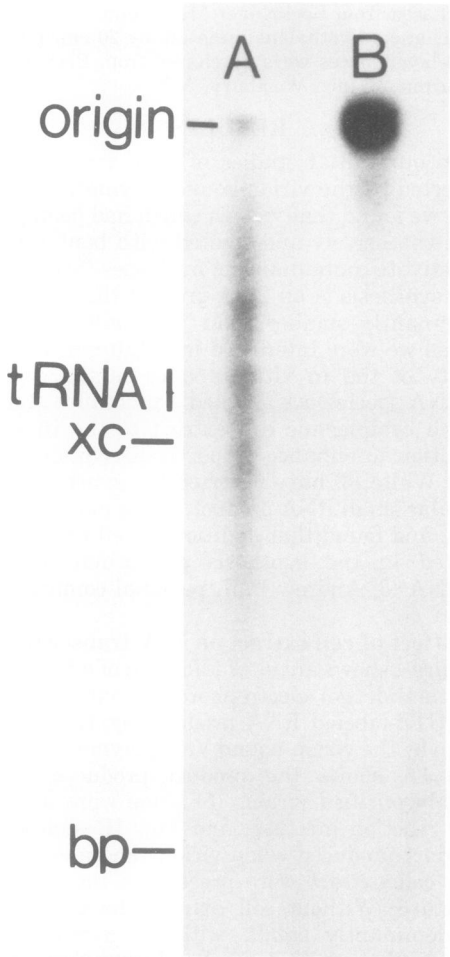

FIG. 1. *Autoradiogram of a gel electrophoretic analysis of VSV mRNA synthesized in vitro. VSV mRNA was synthesized in vitro ([α-32P]GTP label) in the absence (A) or presence (B) of cytoplasmic cell extract. Reactions contained S-Ado-Met and were as described in the text, except that the reaction without cell extract was started by the addition of $MgCl_2$ to a final concentration of 5 mM. Electrophoresis was on a 12% polyacrylamide gel containing 7 M urea as described in the text. bp, Bromophenol blue; XC, xylene cyanol FF.*

centrifugation of the virions. Furthermore, ^{32}P-labeled VSV mRNA's purified from VSV-infected cells were not degraded in reactions lacking cell extract and which produced small product RNA. Thus, it appears that if nucleases are responsible for the production of small product, they act only on the in vitro product, perhaps by being localized inside the virions. The cell extract may provide nuclease inhibitors or alternatively may provide positive factors that promote elongation of the transcripts.

Additional experiments attempting to increase the product size were carried out with all four triphosphates at concentrations of 1 or 2 mM, but no change in the size distribution of product made without cell extract was observed. Use of purified ribonucleoprotein cores instead of whole virions in the reaction also had no effect on the size distribution of the transcripts.

Effect of S-Ado-Hcy on the size of the product RNA. To obtain further information on the size of the VSV product RNA synthesized in the presence of cell extract, we analyzed the product on formamide-polyacrylamide gels, a system that we have used previously to analyze and purify the VSV mRNA's synthesized in VSV-infected cells (20, 27). Figure 2A shows an autoradiogram of a gel electrophoretic separation of poly(A)-containing mRNA synthesized in VSV-infected cells. Figures 2B and C show the poly(A)-containing RNA products synthesized in vitro in the presence of *S*-Ado-Met and *S*-Ado-Hcy, respectively. The RNA synthesized in the presence of the methyl donor *S*-Ado-Met contains products that comigrate with VSV mRNA bands 2, 3, and 4, whereas the RNA synthesized in the presence of the methylation inhibitor *S*-Ado-Hcy is heterogeneous in molecular weight but predominantly larger than band 3 mRNA. The absence of discrete VSV mRNA species in the unmethylated RNA product as well as the large size of the product suggested that methylation might play a role in generating the proper size of transcript, perhaps by providing recognition sites for cleavage enzymes.

Fingerprint analysis of the methylated and unmethylated RNA. To determine the relationship between the nucleotide sequences present in the discrete methylated and heterogeneous unmethylated product RNAs, we labeled the in vitro product with [α-^{32}P]GTP in the presence of *S*-Ado-Met or *S*-Ado-Hcy and analyzed RNase T1 digests of the RNA products by a two-dimensional fingerprinting system. RNase T1 cleaves at guanosine residues and leaves a guanosine 3′-monophosphate end on each oligonucleotide. Thus, all oligonucleotides generated by T1 RNase cleavage are labeled, with the exception of those that originate from the 3′-terminus of RNA molecules not having a 3′-terminal guanosine. The fingerprints of the methylated and unmethylated RNAs are shown in Fig. 3. At both 15 min and 2 h of synthesis, the fingerprints of the methylated and unmethylated RNAs show the same pattern of oligonucleotides, with the exception of two oligonucleotides that are most obvious in the RNA products obtained after 15 min of synthesis. Oligonucleo-

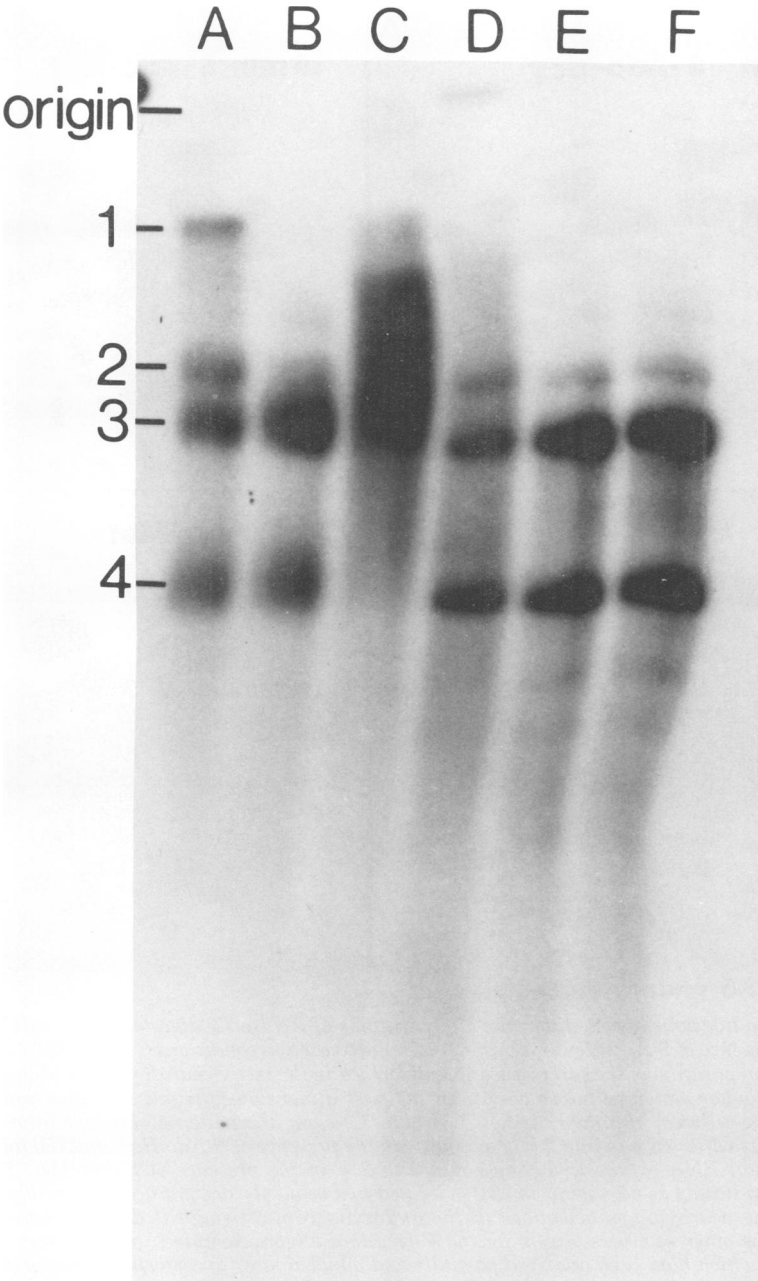

FIG. 2. *Autoradiogram of a formamide-polyacrylamide gel (4% acrylamide) electrophoretic separation of the VSV mRNA's synthesized in vitro and in vivo and treated with RNase H to remove poly(A). Reaction conditions ([α-^{32}P]GTP label) were as described in the text. The figure shows VSV mRNA purified from VSV-infected cells (A), synthesized in vitro in the presence of cell extract and S-Ado-Met (B), and synthesized in vitro in the presence of S-Ado-Hcy (C). (D), (E), and (F) contain the same respective RNAs from which the poly(A) was removed with RNase H prior to electrophoresis.*

tide m1 is present in the fingerprint of methylated VSV RNA but not in the unmethylated RNA, whereas oligonucleotide 1 is present in the fingerprint of unmethylated RNA but not in the methylated RNA. The studies outlined below indicate that these are the methylated and unmethylated forms of a capped 5'-terminal T1 oligonucleotide.

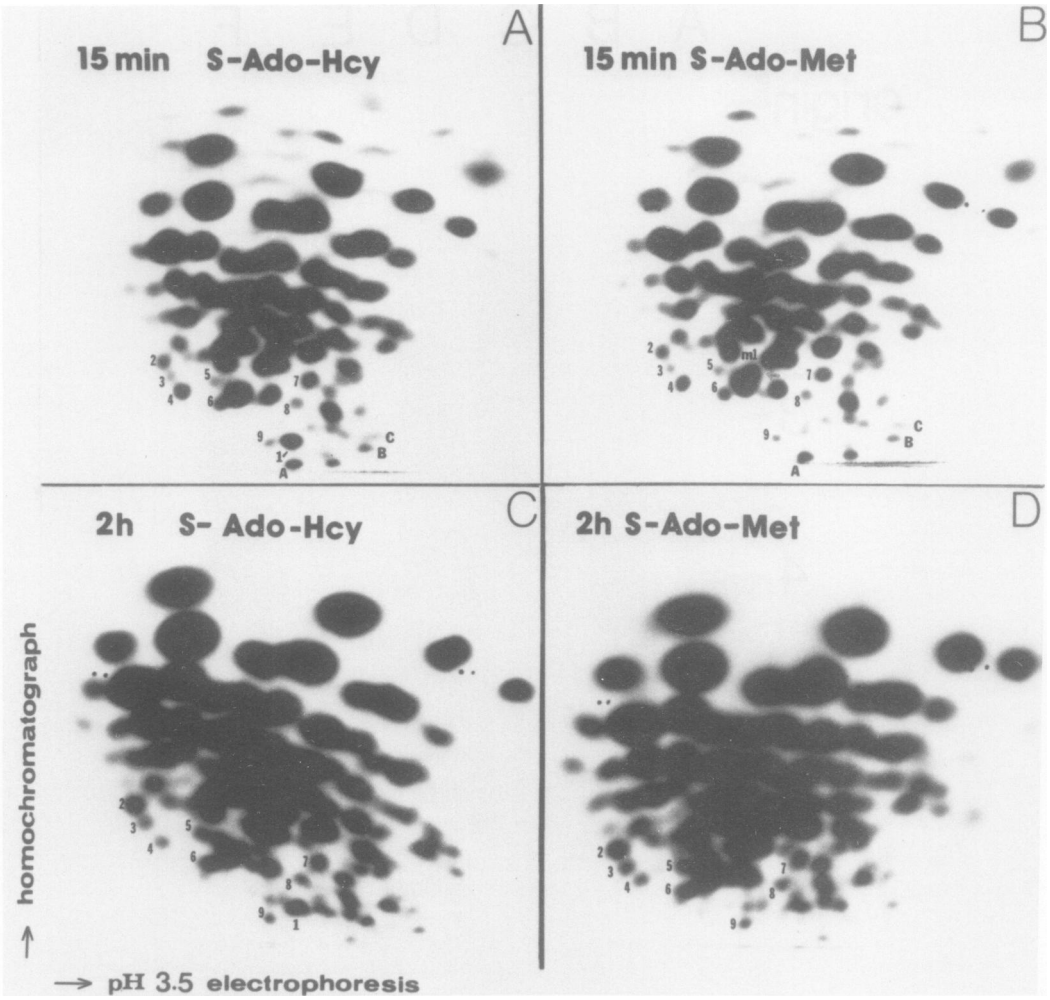

FIG. 3. *Autoradiograms of two-dimensional separations of RNase T1 digests of VSV mRNA synthesized in vitro in the presence of S-Ado-Hcy or S-Ado-Met. In vitro reaction conditions ([α-^{32}P]GTP label) containing cell extract and preparation of the samples were as described in the text. Separation of the oligonucleotides was by pH 3.5 electrophoresis on cellulose acetate in the first dimension followed by homochromatography on polyethyleneimine-cellulose thin-layer plates. (A) and (C) show the patterns obtained from the total RNA synthesized in vitro after 15 min and 2 h of synthesis in the presence of S-Ado-Hcy, and (B) and (D) show the patterns from total RNA synthesized for 15 min and 2 h in the presence of S-Ado-Met. The 5'-terminal oligonucleotide indicated as m1 (arrow in [B]) is located just below the designation on the fingerprint and was resolved from the nearby oligonucleotides in the autoradiogram although it does not appear to be in the reproduction. The other numbers, except for the 5'-terminal oligonucleotide 1, are arbitrary designations of oligonucleotides which have been analyzed partially and which appear to correspond among the fingerprints.*

When digested with RNase A, the T1 oligonucleotides m1 and 1 each gave two labeled products, indicating that they contained two guanine nucleotides instead of one, which is expected from the specificity of RNase T1. One of these products, A^{32}pGp, was common to both oligonucleotides, and its structure was confirmed by mobility on DEAE paper (pH 3.5 ionophoresis) as well as digestion with RNase T2 which gave A^{32}p. The other RNase A products from m1 and 1 had very slow mobilities on DEAE paper ionophoresis at pH 3.5, that from m1 being identical to that of m^{7}G$^{5'}$ ppp$^{5'}$ (m)AmpApCp (26) and that from 1 being slightly slower. This slower mobility is consistent with an unmethylated form of the same oligonucleotide lacking the positive charge contributed by m^{7}G. Further evidence that these oligonucleotides did contain pG and pm^{7}G in a cap structure came from digestion of each slowly migrating RNase

A product with penicillium nuclease, which will cleave all 3'-5' phosphodiester bonds in RNA, regardless of base or ribose modifications (16). From each we obtained a single labeled compound from which the ^{32}P was not released by phosphatase and with mobilities consistent with the structures $m^7G^{5'}$ $ppp^{5'}Am$ and $G^{5'}ppp^{5'}A$, the sequence expected from the 5' end of VSV mRNA's (24, 26). Furthermore, digestion of each with venom phosphodiesterase, an enzyme that digests readily from any free 3'-hydroxyl end, gave pm^7G and pG from these compounds. It was also shown that periodate oxidation and β-elimination of the slowly migrating RNase A product from oligonucleotides m1 and 1 removed m^7G and pG, leaving oligonucleotides that when digested with nuclease P1 gave a phosphatase-sensitive product with the mobility of ATP on pH 3.5 DEAE paper ionophoresis. Because periodate oxidation is specific for 2'-3' hydroxyls, this result indicates that the m^7G and G were linked via a 5'-5' triphosphate to A or Am (pppAm and pppA have approximately the same mobilities on pH 3.5 DEAE paper ionophoresis). Thus, oligonucleotides m1 and 1 contain the methylated and unmethylated cap structure and are presumably the 5'-terminal oligonucleotides $m^7G^{5'}ppp^{5'}$AmpApCpApGp and $G^{5'}ppp^{5'}$ApApCpApGp isolated by Rhodes and Banerjee from total VSV mRNA by borate affinity chromatography (25), although additional labels would be required to prove their complete sequence. Thus, with the exception of methylation of the 5'-terminal oligonucleotide, the fingerprint analysis suggested that the same nucleotide sequences were present in the methylated and heterogeneous unmethylated VSV mRNA.

Analysis of the poly(A) in methylated and unmethylated RNA. Because the T1 oligonucleotides present in the heterogeneous unmethylated RNA and the discrete methylated RNA species gave identical patterns, we proceeded to analyze the 3'-terminal poly(A) sequences. In vitro product RNA was labeled with [α-^{32}P]ATP, and poly(A)-containing product was purified by binding to and elution from oligo(dT)-cellulose. We noticed initially that ATP incorporation in the reaction containing *S*-Ado-Hcy was always at least twice that obtained in the reaction containing *S*-Ado-Met, whereas GTP incorporation had been identical in both reactions. To determine whether this discrepancy was due to synthesis of additional poly(A) in the reaction containing *S*-Ado-Hcy, we digested the ATP-labeled products with a mixture of RNases A and T1 that will cleave at U, C, and G but not at A residues. The fraction of this RNA that would then bind to oligo(dT)-cellulose was determined. The oligo(dT)-binding fractions from the methylated and unmethylated samples were 23 and 57%, respectively. These initial results suggested the presence of more poly(A) in the unmethylated in vitro product.

The size of the poly(A) in the product RNAs was then analyzed directly by polyacrylamide gel electrophoresis. First, the [α-^{32}P]ATP-labeled RNAs were digested with a mixture of RNases A and T1 and subjected to electrophoresis on a 12% polyacrylamide gel. A densitometer scan of the autoradiogram of this gel is shown in Fig. 4A. The poly(A) from the methylated RNA has entered the gel completely and has an average size close to that of the 6S (184 nucleotide) marker RNA. In contrast, the majority of the poly(A) from the unmethylated RNA remains at the top of the gel. To obtain further information on the size of the poly(A)

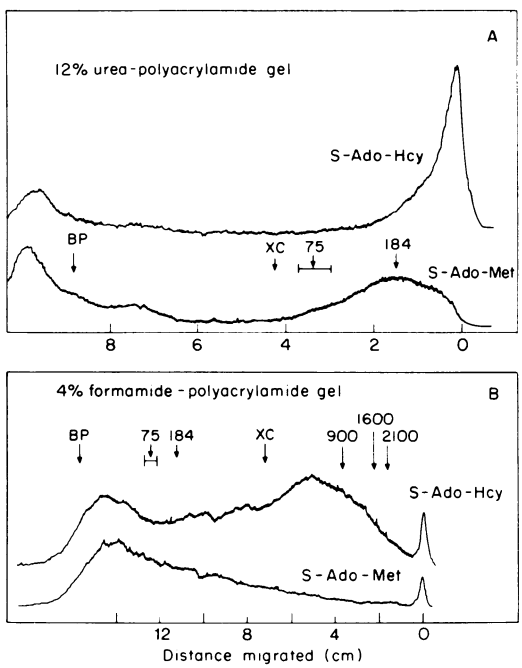

FIG. 4. *Microdensitometer scans of autoradiograms of polyacrylamide gel electrophoretic separations of poly(A) derived from VSV mRNA synthesized in vitro. (A) shows the scan of a 12% polyacrylamide gel of the poly(A) derived from VSV mRNA synthesized in vitro in the presence of S-Ado-Hcy (upper trace) and in the presence of S-Ado-Met (lower trace). The positions of tRNA and 6S RNA markers are indicated in numbers of nucleotides. (B) shows scans of 4% polyacrylamide gels of the same samples of poly(A). The additional markers included on the gel are the VSV mRNA's, with their approximate lengths indicated in numbers of nucleotides. BP, Bromophenol blue; XC, xylene cyanol FF.*

from the unmethylated product RNA, an additional portion of each digested RNA sample was analyzed by electrophoresis on a 4% formamide-polyacrylamide gel. This analysis (Fig. 4B) shows that the poly(A) from the unmethylated RNA is large and heterogeneous, ranging from about 200 to 2,000 nucleotides in length and having an average size of about 700 nucleotides. In contrast, the poly(A) from the methylated RNA is much smaller and does not resolve completely from the mono- and oligonucleotides on this low-percentage gel. All material identified as poly(A) on the basis of resistance to RNases T1 and A was shown to yield greater than 98% AMP after digestion with RNase T2. It should be noted that the radioactivity in the non-poly(A) material is underrepresented in the scans of both the low- and high-percentage gels because of diffusion of the small oligonucleotides and mononucleotides at the bottom of each lane in the gel.

Long poly(A) is covalently linked to VSV mRNA. The above size analysis of the poly(A) present in the unmethylated VSV mRNA product suggested that the poly(A) alone could account for the production of large heterogeneous transcripts containing the mRNA sequences. Direct evidence that this is in fact the sole explanation for the production of the large heterogeneous transcripts was obtained by using RNase H to remove the poly(A) after hybridization of the mRNA preparations to oligo(dT). RNase H will digest the RNA strand of a DNA-RNA hybrid and has been used previously to remove poly(A) from cellular mRNA's (30, 34). Figure 2 shows an autoradiogram of a gel electrophoretic analysis of VSV mRNA's before and after removal of poly(A). Figures 2A, B, and C show VSV mRNA's synthesized in vivo, in vitro in the presence of *S*-Ado-Met, and in vitro in the presence of *S*-Ado-Hcy. Figures 2D, E, and F show these same RNA samples after removal of the poly(A) with RNase H. The most obvious change is in the heterogeneous unmethylated RNA (compare Fig. 2C and F), which is converted after removal of the poly(A) into three discrete RNA bands that comigrate with the methylated, deadenylated derivatives of VSV mRNA's synthesized in vivo (Fig. 2D) and in vitro (Fig. 2E). A slight decrease in RNA band width and an increased mobility are evident after treatment of the other RNAs with RNase H, presumably because of removal of poly(A) size heterogeneity and the small decrease in molecular weight (see also Fig. 5).

A quantitative analysis of these gels was obtained from microdensitometer scans of the autoradiogram (Fig. 5). RNA bands corresponding in position to bands 2, 3, and 4 from RNA synthesized in vivo are clearly visible in both the methylated and unmethylated products after removal of the poly(A). Also, the relative amounts of the different mRNA bands are identical in the methylated and unmethylated products after removal of the poly(A) and are similar to the RNAs obtained from VSV-infected cells, although apparently less band 2 RNA and no band 1 RNA are synthesized. In some other preparations trace amounts of an RNA band comigrating with band 1 mRNA have been visible.

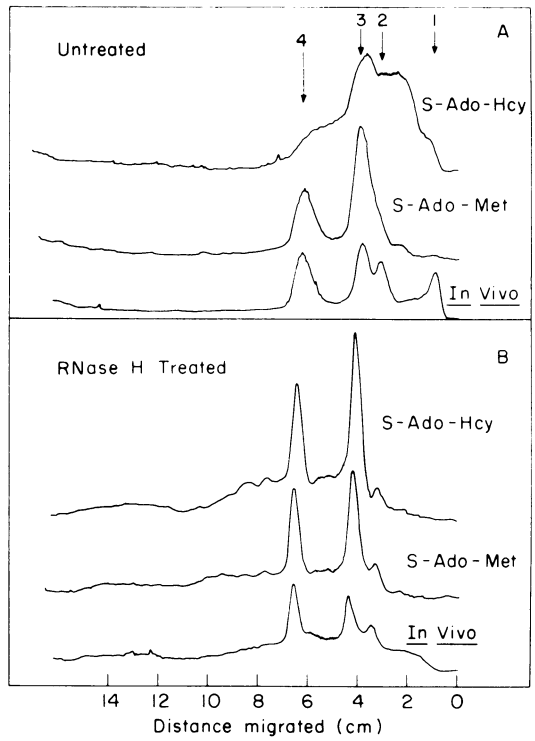

FIG. 5. *Microdensitometer scans of the autoradiogram shown in Fig. 3. The scans of poly(A)-containing VSV RNAs from (A), (B), and (C) of Fig. 3 are displayed in order from the top to bottom of (A). The scans of the poly(A)-minus (RNase H-treated) samples of (D), (E), and (F) of Fig. 3 are displayed in order from the top to bottom of (B).*

DISCUSSION

Current models of VSV transcription. The recent experiments of Ball and White (3) have demonstrated that there is a single start site for transcription of the VSV genome and that the mRNA species are formed in the order N, NS, M, G, and (L). However, it is not yet clear whether the transcriptase terminates tran-

scription of each mRNA species and reinitiates without leaving the template or whether transcription is continuous followed by RNA cleavage to generate the individual mRNA species. Our kinetic analysis of the order of transcription by using oligonucleotides to identify sequences from the individual mRNA species have confirmed that sequences from the N protein mRNA (band 3) are transcribed prior to the appearance of sequences from the other VSV mRNA's. In fact, the fingerprint of the methylated in vitro product obtained after 15 min (Fig. 3B) is identical to fingerprints we have obtained of the N protein mRNA (27), with the exception of the three oligonucleotides labeled A, B, and C which originate from an unstable RNA, 40 to 45 nucleotides in length, that is transcribed prior to N protein mRNA (J. Rose and M. Brock, unpublished data). This RNA may be identical to or part of the "leader" RNA identified by Colonno and Banerjee (11).

Poly(A) addition and sequential transcription. The results presented have shown that large heterogeneous transcripts produced in vitro in the presence of *S*-Ado-Hcy are large because they contain large poly(A). Furthermore, analyses of the appearance of oligonucleotides in N mRNA (Fig. 3 and unpublished data) have shown that *S*-Ado-Hcy does not affect the rate of appearance of oligonucleotides from N mRNA or from the mRNA's transcribed after N mRNA, but only the extent of polyadenylation of the completed mRNA chains. Complete molecules of N mRNA containing poly(A) are detectable after 12 min of synthesis in vitro. At this time, sequences from the other mRNA's are not detectable; thus, addition of poly(A) to N mRNA apparently occurs prior to completion of the sequential transcription of the other mRNA's. These results suggest that during the course of sequential transcription poly(A) is added to the individual mRNA transcripts as they are completed. Because the extent of polyadenylation of N mRNA can be affected greatly without affecting further transcription, presumably different enzyme molecules (although perhaps the same protein species) carry out the continuation of transcription and polyadenylation of the previously synthesized mRNA chains.

Role of the cell extract. We have little information as to what factor(s) in the cell extract is responsible for promoting proper transcription in our system or in the system described by Ball and White (3). The factor is heat labile, but not ribosome associated. One group has reported VSV in vitro transcription of predominantly large mRNA products without addition of cellular factors (6, 9), but we have been unable to reproduce these results.

We have carried out experiments which indicate that the cell extract is not required to obtain the effect of *S*-Ado-Hcy on VSV transcription. In these experiments we observed that the small fraction of intact VSV mRNA produced without cell extract has very long poly(A) when synthesized in the presence of *S*-Ado-Hcy. However, when neither *S*-Ado-Met nor *S*-Ado-Hcy is present, the poly(A)-containing fraction does contain the correct mRNA species. These results suggest that the long poly(A) is generated as a direct result of the presence of *S*-Ado-Hcy, rather than because of the absence of methylation, although we have not ruled out a low level of methylation occurring in the absence of *S*-Ado-Hcy. Methylation does occur in the transcription system with cell extract lacking added *S*-Ado-Met, presumably because *S*-Ado-Met is present at low levels or is generated in this system.

Methylation and poly(A) addition. Since nothing is known about the mechanism by which the length of poly(A) on mRNA molecules is determined in any system, it is interesting that a compound which blocks VSV mRNA methylation also increases the extent of poly(A) addition. Methylation in vitro of both base and ribose groups at the 5'-end of VSV mRNA with *S*-Ado-Met as the methyl donor has been reported, but no methylation at the 3'-end of VSV mRNA or at other positions has been detected (1; J. Rose, unpublished data). Also, we have been unable to detect methylation of any of the VSV proteins in vitro. Thus, the effect of *S*-Ado-Hcy may be through a direct interaction with the poly(A)-adding enzyme.

The average size of poly(A) on VSV mRNA synthesized in VSV-infected cells has been reported as about 100 to 200 nucleotides (15, 31). We have observed variability in this poly(A) size (27), but consistently find that it is at least 30% shorter than the poly(A) on VSV mRNA synthesized in vitro in the presence of *S*-Ado-Met. This size discrepancy may reflect degradation of poly(A) in vivo (29).

Weiss and Bratt (35) have reported large poly(A) on Newcastle disease virus mRNA synthesized in vitro and also observed increased transcript sizes (compared with RNA synthesized in vivo), which might be explained by the presence of very large poly(A). Such discrepancies between in vivo and in vitro transcripts might be eliminated by the presence of *S*-Ado-Met in the in vitro transcription system.

We have been able to translate the unmethylated RNA containing the large poly(A) into

the authentic VSV proteins in an in vitro translation system from rabbit reticulocytes (Lodish and Rose, manuscript submitted for publication). The efficiency of translation was 13% as compared with the methylated RNA containing normal poly(A). Because the RNA containing long poly(A) was also unmethylated, it was not possible to assess the effect of the large poly(A) on translation.

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